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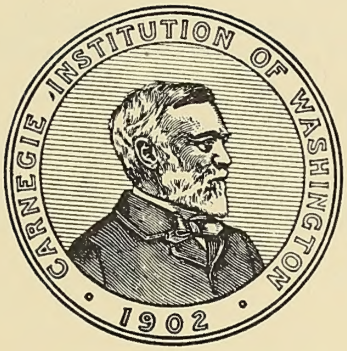

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I. A Cytological and Biochemical Study of the Ovaries of the Sea-urchin *Echinometra lucunter*. By D. H. Tennent, M. S. Gardiner and D. E. Smith. Pages 1 to 46, 7 plates.

II. Observations on the Formation of the Egg of *Echinometra lucunter*. By Ruth A. Miller and Helen B. Smith. Pages 47 to 52, 6 plates.

III. Studies on the Coral Reefs of Tutuila, American Samoa, with Especial Reference to the Alcyonaria. By Lewis R. Cary. Pages 53 to 98, 7 plates, 14 text-figures.

IV. Formed Components and Fertilization in the Egg of the Sea-urchin *Lytechinus variegatus*. By Esther C. Hendee. Pages 99 to 105 2 plates.

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I

A CYTOLOGICAL AND BIOCHEMICAL STUDY OF THE OVARIES OF THE SEA-URCHIN ECHINOMETRA LUCUNTER

D. H. Tennent, M. S. Gardiner and D. E. Smith

With seven colored plates

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A CYTOLOGICAL AND BIOCHEMICAL STUDY OF THE OVARIES OF THE SEA-URCHIN ECHINOMETRA LUCUNTER

INTRODUCTION

The investigations upon which this paper is based constitute a new method of attack on the problem of the functional significance of chondriosomes, Golgi bodies and other "inclusions" in protoplasm.

The method involved the study of formed components of the cytoplasm as *structures* and as *substances*. It demanded the usual study of the tissues, with a discriminating use of fixing fluids and stains, and an analysis of the tissues, with a quantitative separation of the component substances. It demanded, further, the treatment and study of these separated fractions with fixing fluids and stains similar to those used on the tissues, and a comparison of the fixed and stained structures with the fixed and stained substances. It demanded finally a quantitative recombination of the extracted substances with subsequent emulsification, fixation, staining and comparison with the tissues and with the preparations of separated substances.

The methods used have resulted in conclusions that are less speculative in nature than those that have been drawn from the more strictly morphological studies of the last thirty years.

MATERIAL AND METHODS

In 1926 a definite research program for a histochemical and biochemical study of the eggs and ovaries of the sea-urchin *Echinometra lucunter* was begun, a preliminary examination having shown that this material was unusually well adapted to a study of this kind.

Two series of material were prepared. For the material intended for chemical analysis, alcohol was used as a preservative and solvent. For that intended for histological study, the fixing fluids that have proven of value in the study of chondriosomes and Golgi bodies were used, as well as several of the older general histological preservatives.

It was arranged with Dr. Dorothea E. Smith to make the analysis of about 500 grams of eggs and ovaries that had been preserved in alcohol at the Tortugas Laboratory. Miss Ruth A. Miller and Miss Helen B. Smith, graduate students at Bryn Mawr College, worked under direction on some of the histological material and completed, so far as was then possible, their paper, "Observations on the Formation of the Egg of *Echinometra lucunter*," which is now published after emendation made possible by additional material.

At the end of the first year's work it seemed desirable to make use of the experience in methods that had been gained and to repeat the work in a quantitative way.

Three lots of ovaries of *Echinometra* were collected during the last week in July 1927, at the height of the breeding season, for the analytical work. These lots weighed 304.8, 304.42 and 316.6 grams, wet weight, respectively, and each lot was preserved in 1600 grams of 97.5 per cent alcohol. This material served as the basis of the greater part of the work on separated substances, although for some final checking of results an additional lot weighing 304.1 grams was prepared in 1929.

In preparing this material, the sea-urchins were washed and opened by horizontal transection of the test immediately after they were brought from the collecting grounds. The ovaries were scooped from the aboral half of the test with a broad section lifter and dropped into a bottle containing half the alcohol (800 grams). After dehydration, with frequent agitation, for one hour the supernatant alcohol was poured off into a stoppered cylinder and replaced with the remaining half of the alcohol. After dehydration in this for another hour the original supernatant was poured back into the bottle containing the tissue and the second alcohol, and the whole carefully stoppered. Extraction in this alcohol was continued for more than two months, *i. e.* until the analytical work was begun in the following October.

For the histological work several pieces of the same ovary were fixed and mordanted by the Champy-Kull process (Lee's *Vade Mecum*, 9th edition, page 334), and by the Mann-Kopsch method (Lee, page 341), or by Ludford's modified Mann-Kopsch method (Jour. Royal Micros. Soc., 1926, page 108, second process, *i. e.* osmication at 35°C.). Seven pairs of such fixations were made, fixation in Mann's fluid varying from 45 minutes to 3 hours. It should be mentioned that even when osmication is carried on at room temperature at Tortugas, the conditions are approximately those demanded by the Ludford modification, first process, *i. e.* osmication at 30°C. Material was also fixed in Flemming's chromo-aceto-osmic acid, strong formula, and, for the Feulgen reaction, in sublimate-acetic. In the preliminary work material fixed in Bensley-Altmann, Regaud, Flemming-without-acetic and Bouin were also used.

In the extension of the analytical work, samples of the ether-soluble portion of the original alcoholic extract of the tissues and of each of its fractions were sucked or injected into pith, fixed, embedded and sectioned. Pith was used for the purpose of holding the emulsions, during the processes of fixation and sectioning, in conditions that resemble, as closely as possible, those in tissues. All the pith that was used was prepared by peeling, to remove the hard outer layer, and by soaking for 24 hours in ether to dissolve any fatty substances that might be present. It was then dried in air 24 hours before infiltration. The extracted substances were emulsified, and were introduced into the pith either by injection with a hypodermic syringe or by suction with an ordinary filter pump. The latter method gave more satisfactory results, as a greater amount of the substance was carried into the pith cells. For certain phases of the work, droplets of the emulsion fixed as films on cover-slips gave preparations that were both superior and more convenient to make than the pith sections. Parallel fixations were made in every instance by the Champy-Kull and the Mann-Kopsch methods.

The fractions thus extracted and fixed may be classified as follows: (The detailed account of the separation of the fractions is given in Section II.)

Total Fats—The ether-soluble portion of the original alcoholic extract, presumably containing the cerebrosides, phosphatids, cholesterol esters, neutral fats, fatty acids and possibly soaps.

The Cerebroside Fraction—The flocculent white precipitate thrown down when the concentrated ethereal solution was chilled, and which was removed from the ethereal solution by centrifugalization in cold centrifuge tubes. This fraction was redissolved in ether, reprecipitated by chilling, and again removed by centrifugalization.

The Cephalin Fraction—Precipitated from the ethereal solution with warm alcohol and purified.

Lecithin—The lecithin fraction of the ethereal extract, twice precipitated with acetone.

Cholesterol and Fats—The remainder of the ethereal extract after the removal of the cerebroside, cephalin and lecithin fractions, probably including cholesterol and cholesterol esters, neutral fats, fatty acids and soaps.

Cholesterol—Obtained from the cholesterol and fat fraction after hydrolysis with KOH in 96 per cent ethyl alcohol, by repeated extraction with ether.

Mixed Fatty Acids—After the removal of the cholesterol the solution containing the soaps was acidified and the fatty acids extracted with ether and washed until free from mineral acid. This solution of mixed fatty acids was then separated into

Saturated Fatty Acids, and

Unsaturated Fatty Acids—The ethereal solution of mixed fatty acids was dried, dissolved in warm methyl alcohol and the saturated and unsaturated fatty acids precipitated as their lead salts by treatment with lead acetate. The salts of the unsaturated fatty acids were separated from those of the saturated fatty acids by repeated washing with boiling ether. Each of the separated fractions was then decomposed with dilute nitric acid, extracted with ether and the ethereal solutions washed and dried.

Neutral Fat—Made only as a preparation for the purposes of the microscopic work and not as one of the steps in the progressive analytical process. From a portion of the *Cholesterol and Fats* fraction the fatty acids were removed as soaps by titrating with alkali. The cholesterol was then removed by precipitation with 1 per cent solution of digitonin in alcohol and the neutral fat purified and dried.

Pigment—From the ethereal solution after partial crystallization of the cholesterol.

It is important that it should be clear that the substances extracted are from the so-called follicle cells as well as from the eggs.

The substances dealt with in the study are the fats, and to a lesser extent the glycogen content of the eggs and ovaries. Little has been done with the proteids beyond the mere demonstration of their presence in certain bodies in the egg. Nothing has yet been done with the ether non-soluble substances in the original alcoholic extract.

As a check upon the extracted substances, pith was similarly impregnated with purified (Merck's) cholesterol, emulsified with ether, chloroform and water in proportions similar to those used in making the emulsions of the extracted substances. Commercial lecithin was studied in the same way. Emulsions of fresh mutton fat and of Kahlbaum's triolein and oleic acid were also made and studied both in fresh and in fixed preparations.

The two methods of fixation used were those of Champy-Kull and the Ludford modification of the Mann-Kopsch method, all treatments being similar to that given the tissues. After fixation, the material was dehydrated and embedded in 50° to 52°C. paraffin, and sectioned in the usual way. When stains were used, the method followed was that of Champy-Kull, that is, staining with acid fuchsin, thionin, and aurantia after treatment for 15 to 30 seconds with 1 per cent potassium permanganate and 5 per cent oxalic acid. Where extraction of osmicated substances was indicated, the extraction was made with turpentine whose efficacy had previously been tested with mutton fat fixed by the Mann-Kopsch method. It was found in the case of neutral fats that the action of turpentine was that of a solvent for the osmicated substance, although this was not true for all substances blackened by the action of osmium tetroxide.

In addition to that fixed in the pith, small amounts of the extracted substances and of the commercial substances were used as smear preparations and for observations with Nile blue sulphate, Scharlach R, Sudan III, neutral red and Janus green.

The figures illustrating this paper have been made from wash drawings showing the exact colors of the preparations.

SECTION I

STRUCTURE OF THE OVARY AND OÖGENESIS IN ECHINOMETRA LUCUNTER

By D. H. Tennent and M. S. Gardiner

The ovaries of *Echinometra lucunter* are of the racemose type. The wall is of three layers, an outer epithelial layer (cœlomic epithelium), a middle layer containing muscle fibers and connective tissue, and an inner germinal layer, the cells of which may become either eggs or follicle cells (figs. 2, 3 and 4, 6 to 10, and 11 to 14). In most preparations the outer epithelial layer appears as a thin membrane with scattered nuclei, no cell boundaries being in evidence. In tangential sections the wall of an undistended acinus occasionally shows a distinct cellular character (fig. 5). Miller and Smith ('30) have shown that cells of the germinal layer may become oögonia or that they may form nutrient cells which group themselves around the oögonia in a follicular arrangement.

Hamann ('87) in his histological studies of echinoderms described the early appearance of the primordial germ cells and their migration into the saccular outgrowths from the circular genital tube—these outgrowths being the rudiments of the gonads—and the cells that have migrated into them destined not only to form the definitive germ cells, but the germinal epithelium which later on invests the cavities of the germinal glands. These conclusions of Hamann's have been confirmed by later investigators who have studied the origin and development of the genital rachis.

Hamann's figure of the wall of an ovary of a young *Toxopneustes* shows the outer epithelial layer, a connective tissue layer and an inner layer of developing oöcytes and follicle cells. It lacks the muscle layer that is a striking characteristic of the *Echinometra* ovary. The outer epithelial layer in distended acini of *Echinometra* might easily be overlooked, as in these the layer is very thin and the nuclei scattered. However, by careful study, the layer and its nuclei may always be demonstrated.

The ovaries of *Echinometra* are firmer in consistency, possibly because of greater thickness of wall, than those of most other sea-urchins. The ovary never disintegrates at a touch into a mass of eggs as it does in *Toxopneustes, Lytechinus, Salmacis* and other regular sea-urchins. After complete extrusion of eggs the ovaries persist as well-defined structures.

No information on periodicity of reproductive activity in a given sea-urchin seems to be available. It is not known whether the spawning period extends over a matter of days or weeks, or whether an individual having spawned in one year spawns again in successive years. In 1910 the senior author noted that at the approach of full moon in June and July rather large aggregations of *Lytechinus* might be found on the bare sandy bottom, and that after full moon these individuals were found to have spawned, while scattered individuals, picked up from the feeding grounds contained eggs or sperms in abundance. No examination of the empty ovaries in these sea-urchins was made. Both large and small individuals were fully mature.[1]

In the hope of shedding some light on this and related questions, Echinometras were allowed to shed their eggs completely and were then opened and the ovaries removed and fixed. The process of spawning takes about 15 minutes, the eggs pouring out under noticeable pressure. Only rarely will an immature egg be found in the large quantity spawned. In some instances the gonad will be completely emptied and sections will show no free eggs in the lumen, while in other instances contraction has been incomplete and a considerable number of eggs remain.

No emptied ovary was found that did not still show many undifferentiated cells and developing oögonia along the inner wall (fig. 4). In the gonads fixed before the eggs were extruded, and which were from individuals ready to spawn, the same condition was evident (figs. 7, 8, 9, 11, 12). There seems to be no reason for believing that in *Echinometra* there is no production of eggs after a complete spawning.

The question of origin of the germ cells is of much greater importance. It is commonly assumed that the cells forming the inner layer, the germinal layer, of the ovary, are the descendants of the primordial germ cells that are recognizable in the genital ring. Such an assumption is in harmony with established theory.

Certain facts suggest the consideration of the possibility that the cells of the outer epithelium of the ovary contribute to the visible supply of germ cells. The fact that the cells of the outer epithelium are found frequently in mitotic division, while the cells of the inner epithelium (germinal layer) have never been seen in division may, or may not, be of any significance, as only relatively mature gonads have been studied.

The smallest cells of the germinal layer are of the same size and appearance as the cells of the outer epithelium. Nuclei are sometimes found in positions that suggest that they are migrating through the layer of muscle fibers to the inner epithelium. The outer epithelial layer may become very attenuated and its nuclei infrequent and widely separated, a condition which might arise through stretching rather than because of migration of cells.

The Feulgen preparations (fig. 3) show, scattered among the nutrient spherules, many small nuclei that resemble the nuclei of the outer epithelial layer.

All this is suggestive rather than conclusive. A study of growing ovaries in *Echinometra* extending throughout the year should be made.

Miller and Smith have shown that the undifferentiated cells of the germinal layer, after a definite increase in size without structural modification, may develop in one of three ways.

(1) In some of the cells the nucleus disappears and the cytoplasm becomes broken up to form non-granular spheres.

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[1] Both males and females usually spawn readily after being brought into the laboratory. In the height of the breeding season it is even difficult to get them to the laboratory before spawning begins. This, however, may be accomplished by avoiding overcrowding in the containers, and by protecting the urchins against direct, strong sunlight.

During the summer of 1929 when eggs of *Echinometra* were being used daily in connection with another problem, various experiments were made with changes in temperature, light, increased CO_2 content of the sea-water, the addition of freshly shed eggs or sperms to the sea-water in the containers, etc., in an attempt to determine the factors controlling the onset of spawning. All of the experiments were inconclusive. As a general rule, spawning of both males and females began almost simultaneously in from 2 to 2.5 hours after the sea-urchins had been brought to the laboratory. Occasionally, however, there would be a day when no individuals would spawn. Hence the experiments.

Eggs spawned naturally in the laboratory accept fertilization readily and develop normally, while with eggs removed from the ovaries after opening the test only a low percentage of fertilizations has ever been obtained. This has been true for both *Echinometra mathæi* at the Murray Islands in the Torres Straits region and *Echinometra lucunter* at Tortugas.

(2) In other cells, after an irregular fragmentation of the nucleus, the cytosome breaks up into rounded granular bodies.

(3) Other cells become oögonia.

The granular and the non-granular bodies that have resulted from the reformation of the cell contents may be called nutritive bodies. These nutritive bodies group themselves about the oögonia in a follicular arrangement (figs. 1, 2, 3, 4, 6 to 10, and 11 to 14). The cell walls enclosing the groups usually disappear, although a complete wall may persist.

In this material there is no evidence that the nutritive bodies, as such, enter the oögonia. Miller and Smith concluded that there was an actual incorporation of these bodies. In the material that forms the basis of this report the indentation of the surface of the growing oöcyte is often evident (figs. 4, 7, 11, 12), and nutritive bodies sometimes seem to lie within the substance of the oöcyte (fig. 13). Careful focusing in the study of such cases has led to the conclusion that these nutritive bodies lie above the surface of the section and that they have been dragged in by the edge of the microtome knife in sectioning.

Our conclusion is that the substance of the nutritive bodies is absorbed as in digestion.

Figure 1 has been drawn from a longitudinal section of the wall of one of the tubular branches of an ovary. The fixation was in Flemming-without-acetic, the staining by the Kull process. Comparison with figure 13 from material fixed in Champy's fluid and stained by the Kull process is of interest. The small black droplets have been interpreted as fats and the red spheres as lecithin. The yellowish droplets are probably to be interpreted as of mixed proteid and lipid content. As this is a longitudinal section the muscle fibers are visible, here and there, in longitudinal view, just beneath the outer epithelial layer. In figures 11 to 14 they also appear in longitudinal view, while in figures 2 and 4 they are shown in transverse section. It will be noted that the muscle fibers are much more readily seen in the Bouin-Iron-Hematoxylin preparations (figs. 2 and 4), than in those made by the Champy-Kull (figs. 11 to 14), or the Mann-Kopsch (figs. 6 to 10) process.

In sections stained by the Feulgen process and counterstained with light green (fig. 3), the presence of thymo-nucleic acid is indicated in the structures stained red. The similarity in appearance between the nuclei of the epithelial layer and the small nuclei scattered among the nutritive bodies is evident. The larger, sometimes irregular, red masses correspond to the fragmented nuclei that may be seen clearly in preparations stained by the commonly used nuclear dyes. As the fats and lipins are not fixed by corrosive-acetic, the fixing fluid used in the preliminary treatment of the material, these do not appear in the section. The spheres stained green correspond to those remaining in the egg after prolonged alcoholic extraction, whose proteid nature is indicated by their reaction to Millon's reagent or to the xanthoproteic test. It seems probable that the spheres stained green by light green in the Feulgen preparations (fig. 3) are of the same nature as those stained yellow in the Flemming-without-acetic preparation (fig. 1).

CYTOLOGY OF THE GONAD

The tissues fixed and stained by the Mann-Kopsch and the Champy-Kull processes, studied in the light of our results with fixed and stained extracted substances, have enabled us to interpret structure in terms of substance.

In the preparation of the material, seven pairs of fixations were made. For each pair a single gonad was selected and pieces fixed in Mann's and Champy's fluids. After the material was sectioned, mounted and the paraffin removed with xylol, four kinds of completed slides were made. The slide was either mounted in balsam directly after removal from the xylol, (1) *not extracted and not stained* (fig. 11); or (2) extracted in turpentine following removal from the xylol, *extracted and not stained* (fig. 12); or (3) carried down through the graded alcohols and stained, *not extracted but stained* (fig. 13); or (4) extracted in turpentine, taken down through the alcohols and stained, *extracted and stained* (fig. 14). In the arrangement of the figures on the plates, the figure of the extracted preparation lies below that of the unextracted, while the figure of the stained preparation lies to the right of its unstained mate. For purposes of comparison therefore one may either read down, *i. e.* figure 11-figure 12, or across, figure 11-figure 13.

From the standpoint of the descriptive histologist the Mann-Kopsch preparations (figs. 6 to 10) are poorly fixed, while from the point of view of the histo-chemist the preparations are very satisfactory. In figure 6 large blackened spheres are shown forming the follicle of an oöcyte. The progressive increase in blackened content during oögenesis is evident, the probable oögonia against the wall having a relatively small number of granules, the oöcyte showing a greatly increased blackened granular content, the maximum being reached in the oöcyte with coarse granules (fig. 9). Following the coarsely granular condition in the oöcyte is a finely granular condition, this being succeeded by a condition in the extracted but unstained egg in which both yellowish and black granules may be recognized. In the extracted and stained mature egg two kinds of granules, black and red, may be seen.

In addition to the deeply blackened spherules forming the follicle a few yellowish spheres are also evident. In some of these the blackening of the surface may be seen. Figure 7, extraction 3 minutes, and figure 8, extraction 9 hours, show that the effect of continued extraction in turpentine is progressive, but not complete. Blackened rims, crescents, networks and spherules persist in sections that have been extracted for from 48 to 72 hours and longer. The two sections are not fully comparable, as the large oöcyte in figure 8 has stored less deutoplasm than that shown in figure 7. The appearance of the oöcyte in figure 10, extracted 16 hours, shows that at certain stages of development resistance to long-continued extraction is high, a younger oöcyte or a mature egg (compare figs. 8 and 10) containing less of the non-soluble blackened material than do stages midway between the two. Figures 7a and 8a, details of figures 7 and 8 under higher magnification, show resistant blackened rims and networks that are present in the follicle. Blackened rims may also be seen in some of the spherules within the large oöcyte in figure 8.

Figure 9, not extracted and stained, and figure 10, extracted and stained, are of especial interest when compared with the unstained series (figs. 6 to 8). The absence of blackened granular material in the oögonia in figure 9, while bodies of similar size in figure 6 contain many granules, is probably due to the fact that the non-granular condition of oögonia is succeeded by a condition in which coarse non-blackening granules are present. In the unstained material these are brownish yellow in color. In the stained material they are pink. This stage is followed by one in which droplets that blacken in osmic acid become increasingly abundant.

Figure 10 (extracted 16 hours and stained) is reasonably comparable to figure 8 (extracted 9 hours, but not stained). Comparison with our results on osmicated fats, presented later in this paper, warrants the statement that the osmicated fat has been dissolved out of the section and that the permanently blackened structures, rims, crescents, beaded rims, spherules, vesicles and networks are osmicated unsaturated fatty acids. The smaller number of spherules in figures 8 and 10, as compared with 6 and 9, indicates that many of these spherules were droplets of fat; these have been dissolved out completely.

The fuchsinophile bodies remaining (fig. 10) are of two kinds—they are either homogeneous or granular. Reference again to the Feulgen preparation (fig. 3) and to the reaction to Millon's reagent shows that these bodies have a protein basis, while the reaction to the stain indicates a lipid content.

The sections of the Champy material (figs. 11 to 14) are equally instructive in another direction. For general histological purposes this material is superior to that fixed in Mann's fluid. There has been little shrinkage in the material, and the three layers—outer epithelium, muscle layer, and germinal layer—may be clearly distinguished.

The general color tone of the material is brownish yellow. In figure 11 (not extracted and not stained), the mature eggs and an oöcyte show a closely packed brownish-yellow granular content, together with a smaller content of black globules. The nutritive bodies are of two kinds, yellowish brown and black. One of the two oögonia shown against the wall has a few small yellow granules, but no black globules, while the other is nearly non-granular.

In figure 12, extracted in turpentine 15 minutes and not stained, the black globules are lacking, these having been dissolved completely by short extraction in turpentine. The conclusion that these globules are in the nature of fats seems obvious.

In the unextracted and stained sections (fig. 13), the granules in the egg and oöcyte are found to be distinctly fuchsinophile, some being of a more distinct clear red than others. The same thing is true of the nutritive bodies in the follicle. Osmicated fat droplets are shown in the egg and in the follicle. Here and there an intact nucleus, resembling the nuclei in the outer epithelial layer, may be seen, and structures resembling disintegrating nuclei are also evident.

In figure 14 (extracted 8 minutes and stained), the cytological features are the same as in figure 13, except that the black droplets have been dissolved. The clear red granules in figures 13 and 14 are acceptable chondriosomes.

Although it is well known that fat that has been subjected to prolonged osmication is more resistant to extraction in turpentine than fat given shorter osmication, it seems desirable to note particularly the results with this material.

All of the material blackened by osmic acid in the Champy fixation is extracted (dissolved) by turpentine within 8 minutes. Some of that blackened by the Mann-Kopsch treatment is equally soluble, but a residue resists prolonged extraction.

MICROCHEMISTRY OF THE GONAD

The fixed and stained extracted substances were: (1) total fats, (2) cerebroside fraction, (3) cephalin fraction, (4) lecithin, (5) fats and cholesterol, (6 cholesterol, (7) mixed fatty acids, (8) saturated fatty acids, (9) unsaturated fatty acids, (10) neutral fat, (11) pigment.

Purified Merck's cholesterol, lecithin, mutton fat, triolein and oleic acid were also studied. Each of these fractions and substances was prepared by the Mann-Kopsch and the Champy-Kull processes in the same manner as has been described for the tissues. Four kinds of slides were made for each fixation, *i. e.* (1) not extracted, not stained; (2) extracted, not stained; (3) not extracted, stained; (4) extracted, stained.

TOTAL FATS

"Total fats" represents the ether-soluble portion of the original alcoholic extract. The solution was of a deep orange color, and, at room temperature, of the nature of a semi-solid. The material obtained from the analysis made in 1927 was dissolved in ether and shaken with distilled water. After it had been drawn into the pith, the ether was evaporated in a current of air and the pith cut into small pieces which were dropped into the fixing agent. Upon sectioning this material, it was found to be unsatisfactory as it was not in the form of an emulsion.

Similar material, obtained from the 1928 analysis, was dissolved in ether and 2 c.c. of the ethereal solution shaken in a stoppered cylinder with 2 c.c. of distilled water, and then ground in a mortar until the odor of ether was gone. The aqueous emulsion was then sucked into pith and the pith fixed.

The results of the observations on this material are presented in Table 1.

The disparity between these two sets of observations is probably to be explained by the difference in treatment of the 1927 and the 1928 material. In the former the solvent was withdrawn from the pith before fixation, so that the substances were not actually in solution when acted upon by the reagent, while in the material prepared from the 1928 analysis, the dissolved fats and lipides represented the disperse phase of an emulsion whose continuous phase was immediately replaced by the fixative. The pith material was not wholly satisfactory, as it is highly probable that there is a kind of fractional infiltration of the extractives into the pith cells, so that no two slides are entirely comparable in the material, or condition of material, represented in them.

After full consideration we have rejected the 1927 results on the ground that the materials fixed were not in the form of an emulsion.

Logically, the total fats fraction, being the one from which all of the other fractions are derived, is placed first in this account. Actually, it was not studied until after the prolonged examination of its separated fractions. As our interpretation of the emulsions of the total fats fraction and our identification of the substances found in them is based on the study of the separated fractions, we will give at this point only a description of the structures found, and reserve all interpretation until after the presentation of the evidence on which identification is based.

The emulsions were not completely blackened by either the Mann-Kopsch or the Champy-Kull fixations. After either fixation, intensely blackened spherules and rims, both partial and complete, lying in a grayish substrate, may be seen (figs. 15 and 16, Mann-Kopsch, and fig. 21, Champy-Kull). Much of the intensely blackened material is dissolved by turpentine, that fixed in Mann-Kopsch (fig. 17) is resistant to solution, while the Champy-fixed blackened material is readily soluble. The Mann-Kopsch material accepts the fuchsin stain (figs. 18 and 19), some of the clearer vesicles taking the stain well, while the intensely black masses show red over black. The unextracted Champy material shows much greater diversity after being stained (figs. 24, 25, 26). Some of the globules retain their gray color (fig. 26), the gray globules often being surrounded by a heavy or (sometimes) by a beaded, black rim. An occasional spherule showing a clear red stain throughout

Table 1—*Total fats*

Mann-Kopsch	*Châmpy-Kull*
1927	
1. Slides not extracted, not stained:	
Angular colorless or yellowish structures, some with and some without blackened rim. Some large masses, with yellow, almost crystalline groundwork, and linear aggregates of fine black granules twisted over surface. Very rarely, isolated black granules.	Some blackened spherules. Some unblackened crystalline (?) needles and plates. In some cases, superficial blackening of crystalline (?) forms.
2. Slides extracted in turpentine, not stained:	
20 minutes in turpentine. Very little, if any, extraction.	20 minutes in turpentine. No blackened spherules. Needle-like and angular crystalline (?) forms evident, some still retaining a slight amount of superficial blackening.
3. Slides not extracted, stained:	
Oval or circular structures, stained red, some of which contain fine blackened particles. No crystalline (?) forms.	Spherical and irregular dark red masses in which fuchsin stain has been superimposed on osmic blackening. No crystalline (?) forms. Some spherules, staining clearly red with no indication of previous blackening.
4. Slides extracted in turpentine, stained:	
25 minutes in turpentine. No staining evident. Angular yellowish structures present.	20 minutes in turpentine. Unstained crystalline structures again apparent.
1928	
1. Slides not extracted, not stained:	
Very uniform conformation of gray masses, enclosed by partial or complete black rims. Aggregates of apparently empty vesicles, with deeply blackened walls.	Irregular blackened masses, some of which are deep greenish black and some a lighter gray. Some are homogeneously blackened; others, both of the greater and lesser degree of blackening, have more deeply blackened peripheral regions, giving appearance of beaded black rim.
2. Slides extracted in turpentine, not stained:	
2 hours in turpentine. Slight amount of extraction. Structures with homogeneous centers and beaded black rims.	2.5 hours in turpentine. The black and gray masses are removed, leaving groups of black rings of unevenly distributed substance.
3. Slides not extracted, stained:	
Large black masses taking fuchsin stain over osmic impregnation. No evidence in this series of slides of the gray-rimmed structures.	Fuchsin stain superimposed on black substance of irregular masses. Beaded black rings enclosing gray substance. Structures similar to blackened rings but staining red. Small clear red spherules.
4. Slides extracted in turpentine, stained:	
1.5 hours in turpentine. Gray substance which takes no stain and black rods in random distribution. Red droplets of varying size.	Large dark masses gone. Red rings gone. Dark and light red spherules present.

(fig. 26) may be found, although usually these red spherules contain a few black granules (fig. 24), and at times only a red rim remains (fig. 26).

After extraction the Mann-Kopsch material, when stained, shows both gray globules and black rods, as well as red droplets of varying size (fig. 20), while the Champy material shows many dark and light red spherules (figs. 28 and 29), as well as some gray spheres with blackened rims (fig. 27).

CEREBROSIDE FRACTION

The work on the cerebroside fraction was done on new material prepared from ovaries collected in July 1929, the fraction prepared from the 1928 material not being regarded as dependable.

An attempted emulsification with water alone and subsequent infiltration of pith was unsuccessful. Consequently an ether-water emulsion was made.

Droplets of this emulsion were dried on a slide in osmic vapor for 4 hours. They were distinctly blackened in 5 minutes; they were brownish black in half an hour, but did not become really black in the vapor. A slide was then prepared by the Ludford-Mann-Kopsch method after fixation in Mann's fluid for 2 hours. It was stained with fuchsin-thionin-aurantia without extraction. The structure was that of a fine grayish emulsion showing no evidence of the stain.

Streaks of an ether-water emulsion of the cerebroside fraction were air-dried on slides and fixed in Champy's fluid for 24 hours, or in Mann's corrosive-osmic fluid for 4 hours. They were then prepared by the Champy-Kull and the Ludford-Mann-Kopsch processes.

The results of the study of these slides are presented in Table 2.

Table 2—*Cerebroside fraction*

Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	
Grayish vesicles with black rims.	Uniformly gray droplets.
2. Slides extracted in turpentine, not stained:	
Extracted 4 minutes. Blackened rims dissolved, grayish vesicles remaining.	Extracted 5 minutes. Much of material dissolved, some grayish droplets and rims remaining.
3. Slides not extracted, stained:	
Grayish vesicles. Rims of vesicles black. No evidence of stain.	Vesicles in matrix. Both vesicles and matrix have a light pinkish tone. Walls of vesicles slightly deeper in pink coloration.
4. Slides extracted in turpentine, stained:	
Extracted 4 minutes: Grayish vesicular matrix. All black has been dissolved. No evidence of stain.	Extracted 5 minutes: Most of material dissolved. Some grayish droplets and vesicles remaining. No evidence of stain.

The results may be summarized thus: Emulsions of the cerebroside fraction are blackened by the Mann-Kopsch, and are darkened, but not blackened, by the Champy-Kull treatment. The blackened, and the darkened, material is readily soluble in turpentine. The Champy-fixed cerebroside is slightly fuchsinophile.

CEPHALIN FRACTION

The cephalin fraction was obtained from the ethereal extract by precipitation with warm alcohol after the removal of the cerebroside fraction. The precipitate was purified by extraction in warm alcohol to remove the fat, followed by solution in ether and reprecipitation, repeated four times. The product was in the form of a dry grayish-pink powder. After being kept for some weeks the color became grayish yellow.

In view of the fact that there may have been some oxidation of cephalin in the 1928 material, additional observations have been made on the cephalin fraction extracted from the 1929 material.

Cephalin readily forms a colloidal solution in water. An examination of sections made from prepared pith, which had been impregnated with an aqueous solution of cephalin and fixed, made it evident that another method of study must be devised. The Mann-Kopsch material showed somewhat flocculent masses of black. The Champy-Kull material showed a few dense but unblackened areas. There was no evidence of granules in the material from either fixation.

Drops of an aqueous solution of cephalin were injected into small globules of celloidin whose surfaces had been allowed to harden in chloroform. These globules were then fixed, embedded, sectioned and mounted in the usual way. Smears of a solution of cephalin on slides were also fixed and studied, and drops of a thick solution of cephalin were floated on the surface of Champy-Kull fluid, and fixed as films. The results are presented in Table 3.

Table 3—*Cephalin fraction*

Mann-Kopsch	*Champy-Kull*
1. Unstained:	
Sections in celloidin: Spherical globules down to the limits of visibility, deeply blackened by osmic acid. Some linear dark streaks containing black droplets at intervals. Smear: Grayish masses; no globules.	Sections in celloidin: Homogeneous groundwork with brownish granular inclusions. Film: Brownish granular mass.
2. Stained.	
Sections in celloidin: Globules (see 1) were stained pink. Smear: Homogeneous pink masses.	Sections in celloidin: Granules (see 1) stained pink. Film: Granules and floccular mass stained pink; many stained thread-like filaments.

In preparing the sections, prolonged treatment with absolute alcohol must be avoided because of its solvent action on the celloidin. Sections and films were cleared in bergamot oil, washed in xylol and mounted in balsam.

Our study showed that because of the smallness in size of the particles of the disperse phase, no staining reaction of these could be demonstrated. In concentrated or mass suspension, cephalin is blackened by osmic acid and may be stained with fuchsin. It was evident that Mann-Kopsch fixation gave a more exact fixation image than did the method of Champy-Kull.

The cephalin fraction prepared from the 1929 material was about 6 months fresher than that extracted in 1928, *i. e.* it had been extracted and dried, ready for microscopical study 5 months after the tissues were preserved in alcohol, instead of 11 months after preservation.

In making the slides used in the staining tests, a very little of the dry cephalin was placed in a small drop of water on a slide, fixed in osmic vapor for 5 minutes and the slide then transferred to Mann's fluid, or fixed directly in Champy's fluid, the subsequent treatment in both cases being that of the standard procedure. The results of the study of these slides are presented in Table 4.

The observations made on the 1929 material are directly comparable with those made on films and smears of the 1928 material. With all of these it is clear that the fixing fluids came in direct contact with the cephalin solutions. Less confidence concerning the fixation can be felt with regard to the material that was injected into celloidin globules before being placed in the fluids.

The results of the study of the fixed cephalin may be stated thus: Heavy concentrated solutions of cephalin are deeply blackened by Mann-Kopsch (fig. 30);

Table 4—*Cephalin*

	Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	Fine blackened masses with crystalline substrate if solution is not complete.	Yellowish masses.
2. Slides extracted in turpentine, not stained:	Extracted 3 minutes: Grayish-brown substrate, some irregular, blackened masses on top; no definite conformation.	Extracted 3 minutes: Identical with above.
3. Slides not extracted, stained:	Reddish-brown substrate; some blackened masses, some grayish masses.	Fuchsinophile masses; some yellowish pink, others deep rose.
4. Slides extracted in turpentine, stained:	Extracted 3 minutes: Pink substrate with fewer blackened and more grayish masses than 3.	Extracted 3 minutes: Same as 3.

the blackening may be removed by extraction with turpentine, leaving a yellowish-brown substrate with some blackened masses (fig. 31); slides that are unextracted and stained show a reddish-brown substrate with blackened and grayish masses (fig. 32); while extraction followed by staining gives a pink substrate with fewer blackened and more grayish masses (fig. 33).

Solutions of cephalin are not blackened by Champy-Kull but persist as yellowish masses (fig. 34); no change is observable following extraction in turpentine; both the unextracted and the extracted slides take the fuchsin stain (fig. 35).

When a few grains of powdered cephalin are placed in a small drop of water on a slide and examined under the microscope the immediate formation of many small gas bubbles may be observed. These bubbles are fixed and blackened in the subsequent treatment of the slide, and may readily be mistaken for solid globules of blackened substance.

The 1929 cephalin was found to be more reactive than the earlier material, thus confirming the belief that some oxidation of the 1928 material had occurred.

The staining capacity of cephalin, together with its chemical nature, suggested the possibility of relation to chondriosomes, and various experiments in staining with Janus green and other vital dyes were made.

Experiments with Vital Dyes

Hanging drops of a solution of cephalin and Janus green were placed on a cover-slip. With a needle the cephalin solution was dragged to the drop of Janus green so as to form a bridge between the two drops.

(1) Janus green 1:10,000: An intensely stained blue membrane was formed at the boundary between the two solutions, a membrane that was not permeable to either substance.
(2) Janus green 1:20,000: Membrane formed at boundary of solutions of cephalin and dye. Stain less intense than in 1:10,000.
(3) Janus green 1:40,000: Bluish streaks not resolvable into granules were formed.
(4) Janus green 1:80,000: No effect evident.

When solutions of cephalin and Janus green 1:10,000 were mounted as a mixed drop under a cover-slip, the same type of precipitation was observed, the membrane having the appearance of deeply stained blue filaments made up of granules. Mounted in this way, the center of the solution of cephalin gradually took on a pinkish color. There never was a return to the condition of the leucobase of the dye.

Through the courtesy of Miss O. M. Fowler, it was possible to compare the *Echinometra* cephalin fraction with cephalin freshly prepared from a sheep's brain. It was found that the former was less waxy in texture, more easily powdered and more soluble in water than the latter. When a strong aqueous solution of the sheep brain cephalin was fixed in osmic vapor, the cephalin separated out in the form of short darkened, but not blackened, threads of varying thickness (figs. 36 and 37).

LECITHIN

Lecithin Fraction

The lecithin fraction was obtained from the ethereal solution of the alcoholic extract of the ovaries, after the removal of the cerebroside and cephalin fractions, by the method of Levene and Rolf ('27). It was dried overnight in a vacuum desiccator over P_2O_5. An aqueous emulsion was sucked into prepared pith, fixed, embedded and sectioned.

The preparations were not altogether satisfactory, the study of the slides making it evident that in some instances the emulsion was one of water in lecithin, and in others of lecithin in water. For this reason other preparations were made by fixing drops of emulsions on slides and by forcing an emulsion of lecithin in water with a pipette into drops of celloidin which had been superficially hardened in chloroform. These drops were then fixed in Mann-Kopsch and in Champy-Kull, embedded and sectioned.

The results of the study of the impregnated pith are presented in Table 5.

Table 5—*Lecithin fraction*

Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	
Very black droplets and masses.	Grayish black foam-like structure; changes to golden brown. Walls of droplets dark as though emulsion were water in lecithin.
2. Slides extracted in turpentine, not stained:	
Yellowish crystalline masses.	Extracted 30 minutes. Grayish emulsion; becomes golden brown after a few days.
3. Slides not extracted, stained:	
No effect of stain evident.	Foam-like structure; pink matrix.
4. Slides extracted in turpentine, stained:	
Extracted 20 minutes: Very black droplets and masses; some thin masses show foam-like structure of emulsion. Extracted 6 hours: Aggregates of droplets in black masses. Evident that fuchsin has been retained by the droplets. Extracted 24 hours: Blackened aggregates of droplets, some with clear center and black rim. Rims stained red over black.	Extracted 30 minutes: Globules, droplets and granules deeply stained with fuchsin. Extracted 1 hour: Very dark red masses of droplets.

The observations made on the celloidin drop preparations, presented in Table 6, are essentially similar.

TABLE 6—*Emulsion of lecithin fixed in drops of celloidin*

Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	
Very black small granules, somewhat aggregated.	Finely-granular, grayish-black mass; some thin-walled vesicles.
2. Slides extracted in turpentine, not stained:	
Extracted 15 minutes. Granules gray.	Extracted 5 minutes. Grayish-black color has disappeared. Bright refractive granules and vesicles instead.
3. Slides not extracted, stained:	
No evidence of stain.	Pink granules.
4. Slides extracted in turpentine, stained:	
Extracted 1 hour. Aggregates of pink granules.	Extracted 15 minutes. Well-stained pink granules.

These two sets of observations may be considered together: In the unextracted and unstained Mann-Kopsch preparations there were dense black droplets and masses of droplets (fig. 38). Short extraction in turpentine removed some of the blackening, leaving a rim of blackened material around the grayish or yellowish center (fig. 39); prolonged extraction removed still more of the blackening, leaving granules varying in color from gray to yellow (fig. 40).

The unextracted Mann-Kopsch slides (fig. 41), when stained by the fuchsin-thionin-aurantia process, had the same appearance as the unextracted, unstained emulsions (fig. 39).

No indication of stain can be seen after short extraction. With increased extraction the droplets become increasingly fuchsinophile. The length of time necessary to complete extraction depends on the degree of concentration of the lecithin in the droplets or in the platelets, a droplet representing a dilute solution of lecithin being completely extracted in less than an hour, while platelets and masses of concentrated lecithin withstand extraction for several hours. With proper extraction the drops of the emulsion, after being stained, show a pink matrix containing clear spheres and some red, some red over black, granules. A thin pink rim may surround an otherwise dense black drop.

In the most deeply blackened preparations, it is evident after 6 hours' extraction that fuchsin is being retained by the blackened droplets. In similar preparations, extracted 1 hour and stained (fig. 42), many pink droplets may be found. Some of the larger droplets show the pink coloration in their more attenuated and therefore more completely extracted regions, while the thicker and less completely extracted portion of the drop remains black (fig. 42).

With Champy-Kull fixation the unextracted and unstained slides show at first a grayish-black, foam-like structure. This color fades out after about a fortnight, the final appearance being that of a yellowish vesicular mass (figs. 43 and 44). When the preparations are extracted in turpentine the droplets lose their original grayish-black color and persist as gray structures (fig. 45). The unextracted preparations have a bluish-red color after the Champy-Kull stain (fig. 46), while the extracted slides show an orange-red color (fig. 47) after similar treatment.

As a general conclusion, drawn from the study of the lecithin fraction, it may be stated that when lecithin is blackened by osmic acid, the blackening may be removed by extraction in turpentine, leaving a residue that will stain with fuchsin.

COMMERCIAL LECITHIN

As a check on the extracted lecithin, purified Eastman's lecithin (practical grade) was emulsified with ether, chloroform and water, sucked into pith, embedded and sectioned. The results of the examination of this material are given in Table 7.

TABLE 7—*Commercial lecithin*

	Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	Small evenly blackened spheres and masses.	Small yellowish crystalline masses. Some black spherules.
2. Slides extracted in turpentine, not stained:	Extracted 10 minutes. Small evenly blackened spheres. Masses have become lighter in color. Extracted 30 minutes. Small evenly blackened spheres. Masses have assumed appearance of groups of yellow platelets.	Extracted 10 minutes: The same appearance as 1. Extracted 30 minutes: Number of yellow platelets and drops has increased, although some black drops are still present.
3. Slides not extracted, stained:	Same as 1, but stained red over black.	Small spherules stained uniformly with fuchsin; larger ones with rim more deeply stained than central region.
4. Slides extracted in turpentine, stained:	Short extraction: Small spheres stained red over black. Longer extraction (75 minutes). Spheres stained red; masses of platelets pink over yellow.	Short extraction (15 minutes). Rods and granules stained slightly deeper than in the unextracted slides. Longer extraction. Some granules and droplets clear red; some droplets black with red rims.

The study of the slides made it evident that two phases of fixed lecithin were represented; one a solution of lecithin in chloroform or ether, the other an emulsion of lecithin in water, the lecithin having come out of solution because of the evaporation of the solvents. The dissolved lecithin appeared in the fixed preparations as small spheres, the undissolved as platelets.

The dense black spherules and masses in the unextracted, unstained Mann-Kopsch preparations of commercial lecithin (fig. 48) showed two types of reaction to extraction in turpentine. The spherules, representing fixed dilute solutions of lecithin, were rather readily extracted and stained (fig. 52), while the aggregates of granules and droplets of more concentrated solutions were more resistant (figs. 49 and 50). In these, an extraction of 30 or more minutes was required before enough of the blackened material was dissolved out to show the yellow substrate. The unextracted slides, when stained, showed red over black (fig. 51), while the extracted slides, after staining, showed red droplets (fig. 52) for the fixed droplets of dilute solutions of lecithin (fig. 52), and yellow, or pink over yellow, for the aggregates of granules (fig. 53).

In the Champy-Kull preparations the undissolved lecithin has a brownish-yellow color, while that in solution is blackened (fig. 54). On extraction with turpentine the brownish-yellow lecithin becomes lighter yellow in color (fig. 56), while the dissolved lecithin (fig. 55) is more resistant to extraction. The conclusion that undissolved lecithin is at most only superficially blackened by Champy, while lecithin in ethereal solution is blackened throughout, seems warranted. Both the undissolved lecithin (fig. 57) and the lecithin in solution (fig. 58) are fuchsinophile, the color in the latter becoming more evident as the black is extracted.

The results of our study of the lecithin fraction from *Echinometra* and the commercial product are in agreement, except that the granules in the sections made from the injected drops of celloidin, fixed in Mann-Kopsch, extracted, but not stained, were gray instead of yellow. Probably they would have become yellow with longer extraction.

It is evident that lecithin stains red with acid fuchsin after Champy-Kull fixation, and similarly after Mann-Kopsch, although in the latter the red of the fuchsin is superimposed on the osmic blackening. The fact that the blackening produced by osmic acid may be extracted in turpentine is of importance.

FATS AND CHOLESTEROL

Fat and Cholesterol Fraction

(Total fat minus cerebroside, cephalin and lecithin fractions)

(*a*) *An aqueous emulsion was made from an ethereal solution of the deep orange-yellow fat and cholesterol fraction.* This was sucked into prepared pith, which was then fixed, embedded and sectioned. The results of the observations are presented in Table 8.

Table 8—*Fat and cholesterol fraction*

	Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	All droplets very black.	All droplets very black.
2. Slides extracted in turpentine, not stained:	Short extraction leaves grayish-brown border containing fine black granules, center of droplet remaining solid black.	Sections of droplets show dark, not black, rim. Center gray; granules, slightly darker than gray matrix, scattered through central substance.
3. Slides not extracted, stained:	Droplets very black; no evidence of stain.	Very black droplets.
4. Slides extracted in turpentine, stained:	Same as extracted, unstained (2).	Extracted 28 hours; dark rim, gray centers, small holes in centers, no granules. Extracted 48 hours; rims thinner than in 28-hours' extraction.

The facts may be summarized as follows: In the Mann-Kopsch fixation the droplets are deeply blackened by osmic acid, they are not stained by fuchsin-thionin-aurantia; their granules and their centers are resistant to extraction. In the Champy-Kull fixation the blackening is less deep than in Mann-Kopsch; the droplets are not stained by fuchsin-thionin-aurantia; their rims are more resistant than their centers to extraction; their granules are completely extracted (dissolved) in turpentine.

In the Mann-Kopsch fixation all of the droplets of the emulsion are deeply blackened (fig. 64), although in some the blackening is superficial, the surface of the droplet being of a dense black while the interior is lighter in color. Short extraction in turpentine (fig. 65) removes much of the blackened material from the surface layers, leaving a core of deeply blackened substance surrounded by a gray cortex containing numerous densely blackened granules. Prolonged extraction (fig. 66, extraction 24 hours) leaves a grayish residuum containing many black granules and small droplets (fig. 66). A thin layer of superficial blackened substance often persists. Neither the unextracted and stained (fig. 67) nor the extracted and stained slides show any evidence of the stain.

With Champy-Kull fixation there is complete blackening of the droplets (figs. 68 and 69), much of which may be extracted quickly by turpentine (fig. 70), leaving blackened granules and droplets in a grayish substrate, but occasionally blackened structures may resist extraction for more than 48 hours (fig. 71).

(*b*) *Aqueous emulsion made from residue after drying ethereal solution over $CaCl_2$.* The ether was evaporated from an ethereal solution of fats and cholesterol by drying over $CaCl_2$. The residue, somewhat more solid than butter in consistency, was then emulsified by rubbing up with a small amount of water in a mortar. Part remained as an emulsion, part quickly separated as large globules of orange-colored oil; apparently the pigment became concentrated in the unemulsified fat. Drops of the emulsion were fixed on slides in Mann-Kopsch and Champy-Kull. The results are presented in Table 9.

TABLE 9—*Aqueous emulsion of concentrated ethereal solution of fat and cholesterol fraction*

Mann-Kopsch	*Champy-Kull*
1. Slides extracted in turpentine, unstained:	
Extracted 45 minutes: Gray matrix containing dark-gray granules, black granules and black-rimmed vesicles (fig. 73). Extracted 24 hours: Gray matrix with centrally placed dark mass, or with small black granules and black rimmed vesicles. Peripheral "husk-like" edge probably the closely stuck rim of drop from which center was lifting away (figs. 74 and 75).	Extracted 20 minutes: Clear matrix with flocculent grayish masses; some with black granules or with granular filaments; some vesicles with a black rim.
2. Slides extracted in turpentine, stained:	
Extracted 24 hours: Same as unstained.	Extracted.

The results may be summarized thus: Drops of fat and cholesterol are deeply blackened by both Mann-Kopsch and Champy-Kull fixation. The Mann-Kopsch material is resistant to extraction (solution) in turpentine: the Champy-Kull material is readily extracted (dissolved). The material does not stain with fuchsin-thionin-aurantia.

(*c*) *Aqueous emulsion made from the residue remaining after drying the ethereal solution over $CaCl_2$, stained with vital dyes.* Two sets of experiments were made, one with neutral red and one with Janus green.

(1) NEUTRAL RED

A droplet of the emulsion made as described under (*b*) was connected with a droplet of 1:20,000 neutral red by the hanging-drop method. The preparation seemed overstained. A similar preparation was therefore made with 1:40,000 neutral red. This concentration gave uniformly good results.

A preparation made in this way shows (1) the continuous phase of water, (2) large orange-colored droplets that take up the neutral red intensely, but remain orange-red in color, and which may enclose smaller droplets that are stained very deeply, (3) smaller droplets ranging in size from the limits of visibility to a diameter about half as great as that of the orange-colored droplets, staining a deep, clear red, (4) a viscid material in scattered, irregular masses, or in small spherules, that attached themselves to the surface of the stained droplets, forming an envelope about them which stained lightly, and (5) numerous air bubbles enclosed by a film of stained fat.

(2) JANUS GREEN

Droplets of the same emulsion were stained in the same manner with 1:20,000 and 1:40,000 Janus green. The results were essentially the same as those with neutral red. The large yellow droplets did not become as blue as the smaller, directly because of their size, indirectly because of their greater content of the orange-yellow pigment, the mixture of yellow and blue giving them a green tone. The same four types of material seen in the neutral red preparations were evident.

(*d*) *Tests of the emulsion made with Nile blue sulphate, Scharlach R and Sudan III.* Nile blue sulphate stained all of the globules blue. Lorrain Smith ('07) found that Nile blue stains neutral fats red and fatty acids blue. There were no red globules following the Nile blue sulphate stain. The globules were either blue or unstained. Scharlach R and Sudan III stained similar globules red, but not intensely so. These seemed to be the globules that were stained red by neutral red. As an *indicator* this should denote the acid nature of the globules. The evidence gave no indication of a neutral fat content in our mixture. We concluded that the material giving the intense blue reaction either represented the substance as found in the tissue, *i. e.* that the fats are fatty acids, or else that these were the hydrolytic products of true fats, produced by the methods of extraction.

At the time that these experiments were made, the analytical work had not proceeded beyond the separation of the fat and cholesterol fraction. It did not seem probable either that all of the fats present in the extracted material were in the form of fatty acids, or that all of the neutral fat had been transformed to fatty acids. Pending the opportunity of an attempted separation of the fat and cholesterol fraction into neutral fat, fatty acids, and cholesterol, two types of experiment were undertaken. One dealt with the reaction of known mixtures of neutral fat and fatty acid to Nile blue sulphate, the other was concerned with the probability of extracted neutral fat hydrolizing, on standing, to fatty acids and glycerine.

Experiments with Mixtures of Triolein and Oleic Acid

The results obtained by staining aqueous emulsions of the extracted fat and cholesterol fraction with Nile blue sulphate made it desirable to try the effect of this stain on known mixtures of triolein and oleic acid. Emulsions of these mixtures were made in water, in the following proportions, placed on slides and stained with Nile blue sulphate. (Oleic acid stained blue; triolein red.)

(1) 10 drops triolein plus 5 drops oleic acid plus 2 c.c. H_2O. All droplets stained blue.
(2) 10 drops triolein plus 3 drops oleic acid plus 2 c.c. H_2O. All droplets stained blue.
(3) 10 drops triolein plus 1 drop oleic acid plus 2 c.c. H_2O. All droplets stained blue.
(4) 20 drops triolein plus 1 drop oleic acid plus 2 c.c. H_2O. All droplets stained blue.
(5) 5 drops triolein plus 2 c.c. acetic-acid-sodium-acetate buffer, pH 4; droplets of triolein stained pink (rose color).
(6) 5 drops triolein plus 2 c.c. acetic-acid-sodium-acetate buffer, pH 5; droplets of triolein stained pink (rose color).
(7) 10 drops triolein plus 1 drop butyric acid plus 2 c.c. water; droplets of triolein stained pink (rose color).
(8) 5 drops triolein plus 1 drop butyric acid plus 2 c.c. water; droplets of triolein stained pink.
(9) 5 drops triolein plus 3 drops butyric acid plus 2 c.c. water; droplets of triolein stained pink (rose color).
(10) 5 drops triolein plus 5 drops formic acid plus 2 c.c. water. Drops of triolein stained pink.
(11) 5 drops triolein plus 2 drops glacial acetic acid plus 2 c.c. water; droplets of triolein stained pink (deep pink).
(12) 1 drop oleic acid in 8 c.c. water; shaken; 2 c.c. of this aqueous emulsion (containing 0.25 drop oleic acid) mixed with 40 drops triolein (ratio 1 part oleic acid to 160 parts triolein); initial reaction, droplets stained pink, followed by a gradual change to bluish pink.

(13) 1 drop oleic acid in 50 c.c. water; shaken; 2 c.c. of this emulsion (containing 0.04 drop oleic acid) mixed with 40 drops triolein by vigorous shaking (ratio 1 part oleic acid to 1000 parts triolein); initial reaction droplets stained pink, followed by a gradual change to bluish pink. At end of 20 minutes the large droplets remained pink, while all of the small droplets had become distinctly blue. As a check on this, an emulsion of 40 drops triolein in 2 c.c. of distilled water was made and a drop of this stained with aqueous solution of Nile blue sulphate. Droplets stained pink and remained pink. There was no development of blue.

Our tests show that, while triolein taken separately is stained red and oleic acid blue by Nile blue sulphate, mixtures containing a very small amount of oleic acid are stained blue by this stain. The blue reaction can not be regarded as necessarily indicating an absence of neutral fat. It seems, however, conclusively to show the presence of a certain type of fatty acid. The tests described above indicate that the blue reaction is not conditioned by hydrogen ion concentration, and demonstrate that it is not given by all unsaturated fatty acids. It was not given by acetic, butyric or formic acids. The reaction is one that suggests further experiments.

Drops of triolein (Kahlbaum) and oleic acid (Kahlbaum) were fixed in watch crystals and on slides by the Ludford-Mann-Kopsch and the Champy-Kull processes. After dehydration the films and slides were placed in turpentine. The results of this experiment are given in Table 10.

Table 10—*Triolein*

Fixed, and extracted in turpentine.

Mann-Kopsch	*Champy-Kull*
Solution began at once; was slow but readily apparent.	Solution began at once. Wholly dissolved in 4 days.

Oleic Acid

Fixed, and extracted in turpentine.

Mann-Kopsch	*Champy-Kull*
No extraction in 4 days.	Solution began at once. Partly dissolved in 1 hour. Wholly dissolved in 4 days.

The extraction (solution) was much slower on the slides than on the films. The final result, however, was the same in both cases.

Mutton Fat

As a test of the probability of the fatty acid reaction of our fat and cholesterol fraction being due to the hydrolysis of an original neutral fat and cholesterol fraction, studies were made on fresh mutton fat. This, when cut into thin slices and stained on slides with Nile blue sulphate, showed only pink globules.

An ethereal extract of mutton fat was prepared and emulsions of this in water made and stained with Nile blue sulphate over a period of six weeks. The tests always indicated neutral fat; the fatty acid reaction never was obtained.

Mutton fat was fixed by the Ludford-Mann-Kopsch process, embedded and sectioned. Sections 12μ thick were mounted on slides and placed in turpentine. The blackened fat began to dissolve at once, a blackish cloud ascending slowly from the sections. At the end of two hours all of the fat was dissolved, leaving only connective tissue. This was stained by the fuchsin-thionin-aurantia process.

Microscopic Separation of the Fat and Cholesterol Fractions

In an attempt to separate the fat and cholesterol fraction into simpler fractions, the ethereal solution was evaporated to dryness over $CaCl_2$, dissolved in chloroform, evaporated and redissolved twice. The dried residue was then washed quickly with warm alcohol in order to free the fatty material from the crystalline material that had formed as the chloroform solution was evaporated. The test of these crystals for cholesterol was negative.

The alcoholic washing was allowed to evaporate under observation. A stage was reached when globules began to separate out of the solution. Under 4 D these globules could be seen to contain fine needle-like crystals. When the solution containing these was placed on a slide, the crystals were very evident. As the alcohol evaporated they disappeared as though by explosion, very fine clear globules remaining. If evaporation was prevented by placing a cover-slip on the preparation, they retained their form and remained visible for several hours.

When a drop of the alcoholic solution was mixed with one of water, the needle-like crystals retained their form and did not disappear. It was possible to bend them into crescents by slight warming over a flame. On staining with Nile blue sulphate, the crystals were unaffected. The other constituents took the blue stain.

Drops of the alcoholic solution, dried slowly on slides, were placed in 1:250,000 neutral red solution at 38°C. for 24 hours. (Method of Parat for demonstration of the vacuome, Lee, '28, page 353.) At the end of 6 hours, small red globules within larger globules were evident; at the end of 24 hours the small globules were no longer visible, the larger globules having become uniformly stained. The small globules seemed to be those derived from the needle-like crystals.

Drops of the alcoholic washing were mixed with an equal quantity of water, dried with heat and fixed in Mann-Kopsch. When extracted 20 minutes in turpentine, black droplets, derived from the crystals, in a grayish, granular matrix, could be seen (fig. 72). Some of these droplets had a clear halo, formed because the surface of the droplet had been dissolved; some had lost their centers by solution, and some had evidently been completely dissolved. There were some granules and some crescentic rims of vesicles, as well as bent rods that show a slightly distorted form of the needle-like crystals observed in the fixed material, that were resistant to extraction; these were very black.

The emulsion of the concentrated ethereal solution, fixed in Mann-Kopsch and extracted in turpentine 45 minutes (fig. 73), shows much the same thing, although the blackened rods are not present.

The resemblances and the differences between figures 72 and 73 on the one hand and figures 74 and 75 on the other are of interest. The former are of the alcoholic washing; the latter of an emulsion of the concentrated ethereal solution in water.

Figure 77 shows part of a thick drop of the ethereal solution, fixed in Mann-Kopsch and extracted in turpentine for 24 hours. The densely blackened material dissolved slowly, leaving an accumulation of small vesicles with a densely blackened wall and a few black granules. These were not affected by continual extraction for an additional 24 hours.

Droplets of the emulsion of the concentrated ethereal solution fixed in Mann-Kopsch, bleached in solutions of potassium permanganate and oxalic acid and stained with fuchsin-thionin-aurantia, showed the emulsoid structure well (fig. 76), many of the vesicles still showing their blackened walls. No effect of the stain was evident.

In the Champy-Kull fixed and stained emulsion of the alcoholic washing of the concentrated ethereal extract (fig. 78), many unstained crystals of cholesterol, some of the crystals of needle-like form and vesicles lying in a reddish-brown matrix, were evident.

Commercial cholesterol blackens in osmic acid. Cholesterol crystals were seen in the evaporated alcoholic washing, and the dried alcoholic washing dissolved in chloroform gave a weak positive reaction for cholesterol by the Salkowski method. It is therefore possible that some of the blackening may be due to cholesterol or to cholesterol esters. We have no evidence on which to base a distinction between the cholesterol esters and the fatty acids. The doubly refractive character of the globules arising from the needle-like crystals, together with the fact that this character disappeared on warming, suggests that these were cholesterol esters. However, they were not stained red by Nile blue sulphate, although they did show yellowish red after Sudan III.

In view of our results later with the separated fatty acids, it seems probable that the greater part of the persistent blackening is due to the presence of the derivatives of fatty acid crystals, and that a lesser part is due to the presence of cholesterol.

CHOLESTEROL CONTROL

Merck's cholesterol, dissolved in ether and emulsified in water and chloroform, was sucked into prepared pith, fixed, embedded and sectioned. The results of the examination of the sections are presented in Table 11.

TABLE 11—*Cholesterol control*

Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	
Fine blackened rods and droplets. It is evident that the cholesterol is crystallizing out of the ethereal solution. That which remains in solution at edge of crystalline substance is deeply blackened (figs. 59 and 61).	Threads, spheres and granules, not blackened.
2. Slides extracted in turpentine, not stained:	
Extracted 24 hours. Crystalline structure more evident than in unextracted slide, indicating probably that a small amount of cholesterol has been removed by turpentine, but it is not completely removed as is fat. Some blackened material in form of fine rods and granules is collected around (at edge and on top of) the crystals (fig. 60).	No effect (see 1 above).
3. Slides not extracted, stained:	
No fuchsin-stained material.	Fine curved threads and granules stained more deeply than spheres, the latter probably to be interpreted as solvent or emulsifying agent. (Evidence for this statement is obtained from control material in which ether, chloroform and water only were sucked into pith.)
4. Slides extracted in turpentine, stained:	
Extracted 24 hours. No fuchsin-stained material.	No effect.

NEUTRAL FAT AND CHOLESTEROL

A preparation of neutral fat and cholesterol having been made from the extracted fat and cholesterol fraction by removing fatty acids and soaps by titration with alkali, an emulsion was made by shaking a few drops in distilled water. This emulsion was stained with Nile blue sulphate. All of the droplets stained pink.

Hanging drops of the emulsion were fixed in osmic vapor. The droplets blackened at once, the oil droplets (black) separating out as the tip of the clear drop of water. Small drops of the emulsion were also placed on slides and fixed in Mann, Champy and Flemming-without-acetic. All of the blackened material in these slides was removed by extraction in turpentine.

Some of the oil that was solidified on the side of the test tube containing the emulsion was removed and dissolved in chloroform. The Salkowski and the Lieberman-Burchard tests for cholesterol were both positive.

In the solidified portion there is some orange-yellow fluid material that does not take the Nile blue sulphate stain. We have concluded that this is the pigment and that this is the constituent of puzzling nature, previously unidentified in our emulsions.

Small droplets of the neutral fat and cholesterol emulsion were dried on slides and placed in dilute aqueous solution of neutral red (Parat vacuome process). After 4 hours in the stain, the individual droplets at the edge of each drop of the emulsion were stained pink and each contained one or several more deeply stained masses.

Similar drops of a preparation of neutral fat with the cholesterol precipitated as cholesterol digitonate, which had been stained for 20 hours in dilute neutral red, showed the neutral fat unstained and the cholesterol digitonate crystals stained slightly pink.

Cholesterol

Purified extracted cholesterol was obtained from the fat and cholesterol fraction by refluxing with KOH in 96 per cent alcohol and washing repeatedly with ether. The final product was in the form of aggregates of white crystals. These crystals, in chloroform solution, gave the characteristic cholesterol reaction to the Salkowski and to the Liebermann test.

The crystals obtained from the alcoholic solution (fig. 62) were characteristic cholesterol crystals with re-entrant angles, while those from ethereal solution (fig. 63) were of two kinds, either rectangular plates with re-entrant angles or brush-like aggregates of needle-shaped crystals collected around one end of the rectangular plates.

From the experiments with our cholesterol from *Echinometra* and with the commercial product it is clear that cholesterol is fixed, if in solution, by the Mann-Kopsch and by the Champy-Kull process. It also appears that the Mann-Kopsch fixed crystals are more resistant to solution in turpentine than are the unfixed crystals.

Mixed Fatty Acids

SATURATED AND UNSATURATED

After the removal of the cholesterol, the solution containing the soaps was acidified and the fatty acids extracted with ether and washed until free from mineral acid.

Droplets of an ethereal solution of the mixed fatty acids were dried on slides, *i. e.* the ether was evaporated. The slides were then placed in dilute aqueous solution of neutral red (Parat vacuome process). The droplets stained a deep uniform red, the color being the same as in the more deeply stained masses noted above in the fat and cholesterol fraction.

In a concentrated ethereal solution of mixed fatty acids, crystals of needle-like form were abundant. On shaking a droplet of this solution with water in a test-tube the crystals disappeared, a fine emulsion resulting. All of the globules in this emulsion stained blue with Nile blue sulphate.

A droplet of the fatty acid with crystals blackened within 2 minutes in osmic vapor.

A droplet of the fatty acid with crystals was connected with a small drop of aqueous solution of Nile blue sulphate and covered with a cover-slip. The liquid portion of the fatty acid solution at once became blue and contracted into strands. The crystals behaved as though they were hygroscopic, attracting water so that a vesicle with a crescent of fatty acid at one side was formed. The vesicles were like the globules in which crystals were formed, observed in our earlier experiments with an alcoholic solution of the cholesterol and fat fraction. The vesicles and the crystals stained with Nile blue sulphate, the crescents (bent crystals) becoming a very deep violet-blue.

These results suggest that Golgi batonettes may be fatty acid crystals, the fatty acid crystals having separated out of the solution of fatty acids or of the mixture of neutral fat and fatty acids.

An emulsion of the mixed fatty acid solution was made in water and drops of this emulsion fixed as films in Mann and Champy in watch-glasses. The films blackened immediately. They were prepared further by the Mann-Kopsch and the Champy-Kull processes, dehydrated and placed in turpentine. The Mann-Kopsch film was resistant to solution, while the Champy-Kull film dissolved readily.

SATURATED FATTY ACIDS

It has already been noted that the saturated and unsaturated fatty acids were recovered from the fat fraction remaining after the removal of the cholesterol. They were precipitated as their lead salts, from solution in warm methyl alcohol, with lead acetate. The salts of the saturated fatty acids were separated from those of the unsaturated fatty acids by repeated washing with boiling ether, the insoluble salts of the saturated acids remaining as a white sediment, while the washings contained the salts of the unsaturated acids, together with dissolved orange-yellow pigment. Each of these lead salt fractions was then decomposed with dilute nitric acid, extracted with ether and the ethereal solutions washed with distilled water and a solution of Na_2SO_4. The solutions were then dried over Na_2SO_4.

After evaporation of the ether the saturated fatty acids were in the form of a dry, whitish cake of wax-like consistency, while the unsaturated fatty acids were in the nature of a heavy yellow oil.

Pieces of the cake of saturated fatty acids were dissolved in chloroform, the chloroform driven off by evaporation in a current of air, and the white residue dissolved in hot ethyl alcohol. After evaporation in a current of air until the dissolved fatty acids began to separate out of the solution, various experiments were made. (1) Following Escher's cigarette paper method,[1] strips of cigarette paper were wet with the alcoholic solution and after drying were placed in OsO_4 vapor. No browning or blackening occurred. (2) A mixture of 1 c.c. of alcoholic solution of saturated fatty acid, 1 c.c. of water, and 1 c.c. of 2 per cent aqueous solution of OsO_4 was made. A grayish-white flocculent mass separated out, the liquid remaining transparent gray in color. After 16 hours, complete reduction of the OsO_4 had occurred. This, however, is to be regarded as secondary osmication produced by the alcohol in the solution (Handwerck, '98) rather than as a primary osmication due to the fat. (3) Droplets of the alcoholic solution were inverted as hanging drops in OsO_4 vapor. A slight graying of the edges of the crystals was visible after 10 minutes, but there was no blackening, even after prolonged duration in the osmic vapor. (4) Droplets of the alcoholic solution were evaporated to dryness on slides, fixed in Mann's corrosive osmic and completed by the Ludford modification of the Mann-Kopsch process. No blackening occurred.

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[1] Escher ('19) states that Altmann ('90) was the first to use absorbent paper in making tests in this way, and also cites the use of the method by Wlassak, L. Smith and Kawamura.

Chloroform-water and ether-water emulsions were made. Macroscopically these emulsions, when fixed by the Mann-Kopsch process, were gray in color. Microscopically they had the appearance of light-gray vesicles in a black, but not dense black, matrix (fig. 79). This matrix was readily soluble in turpentine. After the turpentine extraction, slate-gray vesicles remained (fig. 80).

The results of similar experiments with alcoholic solutions of unsaturated fatty acids and of the fat and cholesterol fraction show striking differences.

For comparison with the usual preparations on slides, a study of the solutions by the cigarette paper method and by mixture in test-tubes was made. The results were as follows:

Cigarette paper method: papers wet with alcoholic solution, dried and placed in OsO_4 vapor.

(1) Saturated fatty acid—no browning or blackening.
(2) Fat and cholesterol fraction—brown in 3 minutes.
(3) Unsaturated fatty acid—black in 3 minutes.

Test-tube method: mixture of alcoholic solution, water and 2 per cent solution of OsO_4.

(1) Saturated fatty acid—grayish-white masses separated out. The liquid became transparent gray in color in 5 minutes. After 16 hours, a dense black precipitate with clear supernatant liquid had formed; the comparative width (depth) of the two layers was about 1:1.
(2) Fat and cholesterol fraction—yellowish at once on addition of OsO_4. Gradually darkened, becoming greenish black after 30 minutes. After 16 hours a dense black precipitate with gray supernatant liquid had formed; the comparative depth of the two layers was about 1:1.
(3) Unsaturated fatty acid—very black droplets separated out at once; the liquid became black in 3 minutes. After 16 hours there had been a very slight settling of a dense black precipitate from the colloidal solution, leaving a thin layer of clear supernatant liquid; the comparative width of the two layers was about 10:1.

In the test-tube experiments it is possible to compare the initial reaction due to the fatty acids with the secondary reaction due to the alcohol. These statements are summarized in tabular form for ease of comparison in Table 12.

Tests of the ether-water emulsion of the saturated fatty acids with Nile blue sulphate gave characteristic blue droplets. The same test, applied to emulsions made by shaking the crystallizing alcoholic solution with water, gave blue droplets and unstained crystals, as well as amorphous masses of the fatty acids, thrown out of solution by the water. These masses retained enough of the stain to give them a greenish tinge. When a chloroform solution instead of an ether solution of the saturated fatty acids was shaken with water and stained with Nile blue sulphate, the preparation showed a mixture of red and blue droplets. As it was obviously impossible that this was an indication of neutral fat and fatty acid, the probable cause seemed to lie in the presence of droplets of chloroform. A mixture of chloroform and water was therefore made, shaken well, and to a drop of this mixture on a slide, a drop of aqueous solution of Nile blue sulphate was added and the preparation covered with a cover-slip. The droplets of chloroform, under the microscope (fig. 87), looked like droplets of fat stained with Sudan III.

Table 12—*Comparison of initial reaction of saturated fatty acids, unsaturated fatty acids, and fat and cholesterol fraction to osmic acid*

Method	Saturated fatty acid	Unsaturated fatty acid	Fat and cholesterol fraction
Cigarette paper...	No browning or blackening.	Black in 3 minutes.	Brown in 3 minutes.
Test-tube........	Grayish-white masses separated out.	Dense black droplets separated out.	Yellow.
Slides............	Slight graying at edges; no blackening.	Black in 3 minutes.	Brown in 3 minutes.

UNSATURATED FATTY ACIDS

The brief comparison of the results of osmication of saturated and unsaturated fatty acids, made in the preceding section, anticipates very slightly the results of the study of the unsaturated fatty acids now to be presented.

An ethereal solution of the unsaturated fatty acids was dried in a desiccator with P_2O_5 to a heavy yellow oil. This was then dissolved in hot alcohol, and the solution allowed to evaporate in the desiccator until crystals began to come down. Droplets of this concentrated solution were then studied.

Smears of the solution of unsaturated fatty acids blacken almost instantaneously in osmic vapor, the smear fixing to a hard, black film. Drops of aqueous emulsion blacken within 3 minutes, the larger droplets in a coarse emulsion settling to form the tip of a hanging drop.

Drops of emulsion placed on slides were fixed in Mann and in Champy after a preliminary fixation in osmic vapor. These slides were then finished by the Mann-Kopsch and the Champy-Kull processes. The results are presented in Table 13.

Table 13—*Unsaturated fatty acids*

Mann-Kopsch	*Champy-Kull*
1. Slides not extracted. not stained:	
Dense black droplets distributed uniformly through a slightly blackened continuous phase.	A general blackening; some droplets very black, some slate-black with dense black granules.
2. Slides extracted in turpentine, not stained:	
30 minutes' extraction: The slightly blackened continuous phase becomes lighter while distributed droplets remain dense black. Extraction continued to 4 days without effect on dense black droplets.	5 minutes' extraction; droplets seen as very black in 1 have appearance of a gray sponge; former slate-black droplets have become lighter in color 15 minutes; large part of material completely dissolved; "sponges" in separated fragments. 20 minutes; slide nearly macroscopically clean. 24 hours; almost completely dissolved.
3. Slides not extracted, stained:	
No evidence of stain.	No evidence of stain.
4. Slides extracted in turpentine, stained:	
No evidence of stain after either short or long extraction.	No evidence of stain at any time during solution.

It is evident that Mann's solution fixes the emulsions of the unsaturated fatty acids very quickly as there is a very uniform distribution of fine, dense black droplets through the slightly, but definitely, blackened continuous phase of the medium (fig. 81). After 30 minutes' extraction in turpentine this medium on the Mann-Kopsch slide has become lighter in color, but the distributed droplets remain dense black (fig. 82). There is no evidence of stainability with fuchsin in either the unextracted or the extracted slides.

On the slides prepared by the Champy-Kull process there was a general, but not a uniform, blackening of the emulsion. Some of the droplets became very black, some were slate-black with dense black granules (fig. 84). The Champy-fixed material is not resistant to solution in turpentine; 5 minutes' extraction transformed the blackest droplets to a gray "sponge" (fig. 85). The slate-black droplets became lighter in color and irregular in form Within 15 minutes, a large part of the material was completely dissolved, the "sponges" lying in separated fragments. In 20 minutes the slide was nearly microscopically clean, although a ring-like mark showed the outline of the original drop and there were a few fragments within the ring. After 30 minutes all of the former black granules had become slate-gray in color. After 24 hours the outline of the original drop was still evident and traces of a matrix containing a few gray vesicles could be seen. Flemming-without-acetic fixation of the same type of emulsion, followed by treatment with potassium permanganate and oxalic acid, showed that the blackened material may be progressively bleached out (fig. 86).

For the purposes of a comparative summary, the results of the study of droplets of the saturated and the unsaturated fatty acids after fixation in Mann-Kopsch and in Champy-Kull are presented in Table 14.

TABLE 14—*Saturated and unsaturated fatty acids*

Saturated Fatty Acids		*Unsaturated Fatty Acids*	
Mann-Kopsch	*Champy-Kull*	*Mann-Kopsch*	*Champy-Kull*
1. Slides not extracted, not stained:			
Grayish black ++	Gray +	Dense black ++++	Black-gray ++
2. Slides extracted in turpentine, not stained:			
Black dissolved in 30 minutes	Gray dissolved in 30 minutes	Black resists solution. Still present after 4 days	Nearly complete solution in 30 minutes
3. Slides not extracted, stained:			
No stain	No stain	No stain	No stain
4. Slides extracted in turpentine, stained:			
No stain	No stain	No stain	No stain

The unsaturated fatty acid fraction from *Echinometra* ovaries blackens almost instantaneously with osmic acid. After thorough osmication it resists extraction or solution in turpentine.

PIGMENT

The ovaries of *Echinometra lucunter* vary in color from a striking orange-yellow to the lighter cream-yellow that is characteristic of the testes. Usually it is possible to distinguish between ovaries and testes by the difference in color, but occasionally an ovary will be found that can be recognized as such only by microscopical examination of its contents. The living eggs are light yellow in color, but in these again there is a good deal of variation between the eggs of different individuals. There are no large pigment granules. The color is somewhat concentrated at the surface, but extends through the entire egg; fragments of the egg, obtained by crushing, are nearly as yellow as the whole egg.

The pigment is readily soluble in alcohol and in ether. It persisted in all of the supernatant liquids after the precipitation of a given fraction. It has therefore been present in all of the mixtures of extracted substances that were obtained by drying either alcoholic or ethereal solutions.

Table 15—*Pigment*

	Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	Fine blackened granules and small globules in grayish substrate.	Fine blackened granules and small globules in grayish substrate.
2. Slides extracted in turpentine 3 minutes; not stained:	Extraction evident. Larger globules still black; smaller globules clear. Grayish substrate unchanged.	All globules definitely paler than in 1.
3. Slides not extracted, stained:	Many globules red over black, a few clear red; grayish substance unstained.	Red over black globules, a few clear red; substrate stained with fuchsin.
4. Slides extracted in turpentine 3 minutes; stained:	Larger globules red over black, smaller extracted globules clear red. Grayish substrate unstained.	Larger globules red over black, smaller globules clear red. Substrate very slightly stained.

The results may be summarized thus: The extracted pigment is blackened by the Mann-Kopsch and the Champy-Kull treatment; the osmicated substance is extracted by turpentine, leaving a fuchsinophile residue. The grayish substrate, seen after both the Mann-Kopsch and the Champy-Kull treatments, is probably the solvent. This contains many fine blackened granules, some of which are resistant to extraction with turpentine.

EMULSION OF EXTRACTED SUBSTANCES

For purposes of comparison with the stained tissues it seemed desirable to recombine extracted substances in the proportions in which they had been found in the tissues and to fix and stain these.

The substances were taken in the following amounts:

Lecithin	0.230 grams
Cephalin	0.1786
Neutral fat (Triolein)	1.0
Saturated fatty acids	0.035
Unsaturated fatty acids	0.465
Cholesterol	0.184
Cerebroside	0.170

With the exception of the neutral fat, none of which was available and for which triolein was substituted, all of these were the substances extracted from *Echinometra* ovaries. The cephalin, cerebroside and lecithin had been freshly prepared. None of the pigment fraction was added.

The substances were dissolved in ether and 25 c.c. of distilled water added. They were stirred in a deep container with an electric stirrer until a heavy, creamy emulsion resulted. In 20 minutes' stirring the mixture had been transformed to a mayonnaise-like mass, most of the ether having meantime evaporated. Portions of this emulsion were then fixed in Mann-Kopsch and in Champy-Kull. The results of the observations on this material are presented in Table 16.

TABLE 16—*Total emulsion of extracted substances*

Mann-Kopsch	*Champy-Kull*
1. Slides not extracted, not stained:	
Dense black masses; grayish masses with black granules and vesicles; black droplets; grayish droplets.	Black droplets; brown droplets; brownish-gray droplets.
2. Slides extracted in turpentine, not stained:	
a. Extracted in turpentine 3 minutes. Grayish masses with vesicles having dense black rims; black droplets of varying sizes; some black masses.	a. Extracted in turpentine 3 minutes. Blackened substance largely extracted; brown droplets numerous; gray matrix.
b. Extracted in turpentine 30 minutes. All except two of black masses were dissolved off, two small ones remaining. Remainder of emulsion persisted as grayish smear. Under 4 D seen as vesicular matrix, walls of vesicles having black rim.	b. Extracted in turpentine 20 minutes. The slide was macroscopically clean. Under microscope numerous yellowish and dark droplets visible. Matrix dissolved off completely.
c. Extracted in turpentine 30 days. Black rims persisted.	
3. Slides not extracted, stained:	
Dense black masses. Black droplets; red droplets; black droplets with grayish border; grayish masses containing many black granules and a few red granules; pinkish masses containing a black drop or with many black granules. Many small light-pink spheres. Some vesicles with black walls.	Black droplets. Red over black droplets (red border); red droplets. Pink matrix enclosing masses of the above and brown (yellowish-brown) droplets.
4. Slides extracted in turpentine, stained:	
a. Extracted in turpentine 3 minutes.	
Grayish masses with vesicles having dense black walls (rim). Red droplets; red droplets with black center; pink droplets; some black masses.	Black droplets; many brown droplets; many red droplets; patches of pink matrix enclosing the above.
Extracted in turpentine 30 minutes. Grayish vesicular smear. Vesicles with dense black rims. Some red droplets; some pink; many gray.	Extracted in turpentine 20 minutes. Red droplets; pink droplets; red droplets with black centers; red over black droplets; a few black droplets; red vesicles; pink vesicles. No matrix.

The facts may be summarized as follows: The continuous phase of the emulsion was water in which some of the constituents of the emulsion were slightly soluble. This gave the appearance of a gray matrix. In the Mann-Kopsch preparations the droplets in the emulsion were deeply blackened, all of the blackened substance, except the rims of vesicles, being extracted in turpentine. After extraction, many droplets were found to be fuchsinophile. In the Champy-Kull preparations, the blackening was less general and all blackened droplets were extractable. After extraction many fuchsinophile droplets could be seen.

The reactions of the emulsion made from the recombined extracted substances were similar to those made from the total fats fraction and to the preparations made from the fixed tissues.

The study of the emulsion of total extracted substances is especially valuable when considered in connection with the study of fixed tissues. The truth of this statement is evident when it is remembered that the emulsion contains fats and closely related substances, *but no proteins*, while the fixed tissues afford a range of possibilities extending from fixed proteins, *but no fats*, to fixed *proteins and fats*.

It is thus possible to consider the emulsified fat content of the egg and to compare it with the extracted egg in which little more than a coagulated protein basis is left, or with the egg in which both proteids and fats have been fixed.

EXPERIMENTS ON REDUCTION OF OsO_4

Our results make it clear that certain unsaturated fatty acids satisfy the requirements of a Golgi substance in that they reduce OsO_4 and form a black compound whose color is not extracted by prolonged treatment with turpentine. These results, however, do not lead to the conclusion that the Golgi substance is always in the nature of an unsaturated fatty acid.

In consideration of the conditions existing in muscle, in which Golgi substance may be demonstrated, experiments with lactic acid and glycogen were undertaken. It was found that a mixture of lactic acid and glycogen became dark when placed in osmic vapor. It was then found that a 0.33 per cent solution of lactic acid blackened slowly, after some hours, when placed in osmic vapor. This fact led to a series of experiments for showing the effects of reducing sugars on osmic acid. These suggested themselves not only because of the possible connection with the Golgi substance of muscle, but because of the idea that the vacuome of plant cells is the analogue of the Golgi substance of the animal cell.

(1) 1 per cent solutions of both dextrose and lactose, to which was added an equal quantity of 2 per cent osmic acid solution, were prepared. No blackening occurred.

(2) Drops of these sugar solutions were placed on slides and kept in osmic vapor for 24 hours. No blackening occurred.

(3) 5 per cent solutions of both dextrose and lactose were acidified with HCl, osmic solution added, and boiled. No blackening occurred.

(4) 5 per cent solutions of dextrose, lactose and cane sugar were made distinctly alkaline with NaOH, and 2 per cent solution of osmic acid added. The solutions of dextrose and lactose became light yellow on addition of the osmic solution. The cane-sugar solution remained unchanged in appearance. Each solution was then boiled. The dextrose and the lactose solutions blackened quickly. The appearance of the solutions suggested a colloidal solution of osmium. The cane-sugar solution remained unchanged, *i. e.* perfectly clear.

(5) 2 per cent osmic solution was added to an aqueous solution of NaOH. This immediately became light yellow in color. On boiling, the solution became darker, but did not blacken. There was no evidence of reduction of osmic acid.

(6) Droplets of alkaline solutions of dextrose and lactose were placed on slides in osmic vapor at a temperature of 35° C. These darkened in 4 hours and were distinctly black in 24 hours.

The results are suggestive because of their proof that these reducing organic substances blacken (reduce) osmic solutions. They seem to prove conclusively that osmic acid is not specific as an indicator for either Golgi substance or vacuome.

Our studies have shown that a black compound, resistant to extraction in turpentine, is formed between osmic acid and unsaturated fatty acid. This simulates the Golgi substance. It is equally clear that other reducing substances of the cell are capable of reducing osmic acid.

The results are suggestive further because of the fact that reduction of the osmic acid by the sugars occurs only in an alkaline medium. This is very significant when considered in the light of staining reactions.

COMPARISON OF RESULTS ON EXTRACTED SUBSTANCES WITH THOSE ON TISSUES

In the correlation of the results on the stained extracted substances with those on the stained tissues, the authors wish to make it clear that they do not regard their investigations as complete. The only substances studied are those that were contained in the ethereal solution of the original alcoholic extract. It is not possible to say that the substances extracted are those that were in the tissues. To make such a claim it would be necessary to assume that no changes, due to laboratory manipulation, have taken place. That is obviously impossible.

No assertion that the various "fractions" represent pure substances is made. It is in recognition of this fact that the word "fraction" has been used.

Further, it is recognized that few, if any, of these substances are present in a pure form in the tissues. They are soluble in one another and these mixed solutions may be dispersed in a finely divided condition throughout the cytosome, often incorporated in a protein base. Of the substances studied, possibly the neutral fats represent the closest approximation to single pure substances existing in a cell, but even these have a cholesterol content.

Finally, the authors hold no brief for the idea of "specific" stains. A 1:500,000 aqueous solution of Janus green B is said to be specific for chondriosomes; and we have found no evidence contrary to this. Nile blue sulphate has proved itself to be a very dependable indicator for use in distinguishing between pure triolein and oleic acid. Further than that we can not go.

The conclusions that we have drawn are not based on staining reactions alone, but on evidence deduced through an analytical consideration of the chemical nature of known substances and the nature of the reactions of these substances to stains, solvents and preservatives.

In order to facilitate the comparison of results, the facts are presented in the form of a tabular summary. The numerical designations are the same as those used in all of the preceding tables,

1. Not extracted, not stained,
2. Extracted in turpentine, not stained,
3. Not extracted, stained,
4. Extracted in turpentine, stained.

The evidence presented in Table 17 is convincing. Further elaboration seems almost an unnecessary formality.

The essential facts are that in the extracted material prepared by the Ludford-Mann-Kopsch process, non-extractable blackened substance is found only when unsaturated fatty acids are present, and it is possible to stain with fuchsin only when cephalin or lecithin is present, while in the material prepared by the Champy-Kull process, all of the blackened substance is extractable in turpentine, and again it is only when cephalin or lecithin is present that it is possible to stain with fuchsin.

In the tissues prepared by the Ludford-Mann-Kopsch process, there is a non-extractable blackened residuum in the form of granules, networks and rims of vesicles (figs. 7, 8 and 10), and it is possible to stain certain components with fuchsin after the extraction of the turpentine-soluble blackened material. In the tissues

TABLE 17—*Total fats*

	Mann-Kopsch	*Champy-Kull*
1.	Gray masses, black rims.	Gray and blackish masses, black rims.
2.	Slight extraction.	Extensive extraction.
3.	Red over black.	Red, and red over black.
4.	Black rods and red spherules.	Red spherules.
	Cerebroside Fraction.	
1.	Gray and black.	Gray.
2.	Black extracted.	Extensive extraction.
3.	Gray and black; not fuchsinophile.	Slightly fuchsinophile.
4.	Gray, not fuchsinophile.	Not fuchsinophile.
	Cephalin Fraction.	
1.	Black and gray.	Brown and yellow.
2.	Black and gray.	Yellow.
3.	Reddish brown, gray.	Pink and rose.
4.	Gray, black and pink.	Pink and rose.
	Lecithin.	
1.	Black.	Grayish black, golden brown.
2.	Yellow.	Gray, golden brown.
3.	Black, red over black.	Pink, red.
4.	Red over black; pink over yellow.	Red.
	Fat and Cholesterol Fraction.	
1.	Very black.	Very black.
2.	Grayish brown, black.	Dark gray.
3.	Very black; not fuchsinophile.	Very black.
4.	Grayish brown, black; not fuchsinophile.	Gray; not fuchsinophile.
	Neutral Fat and Cholesterol.	
1.	Black.	Black.
2.	Complete extraction of black.	Complete extraction of black.
	Cholesterol.	
1.	Black.	Not blackened.
2.	Slight extraction.	Slight extraction (solution).
3.	Black; not fuchsinophile.	Slightly fuchsinophile.
4.	Not fuchsinophile.	Not fuchsinophile.
	Mixed Fatty Acids.	
1.	Black.	Black.
2.	Resistant to extraction; black residuum.	Complete solution.
	Saturated Fatty Acids.	
1.	Gray vesicles in slightly blackened matrix.	Gray.
2.	Gray vesicles.	Complete solution.
3.	Not fuchsinophile.	Not fuchsinophile.
4.	Not fuchsinophile.	Not fuchsinophile.
	Unsaturated Fatty Acids.	
1.	Black.	Black and slate-black.
2.	Black, resistant to extraction.	Extracted.
3.	Not fuchsinophile.	Not fuchsinophile.
4.	Not fuchsinophile.	Not fuchsinophile.
	Emulsion from Extracted Substances.	
1.	Black and gray.	Black, brown, brownish gray.
2.	Incomplete extraction; black and gray; black rims.	Complete extraction.
3.	Black, pink, red.	Black, brown, pink, red.
4.	Black rims; pink and red droplets.	Pink, red.
	Fixed Tissues.	
1.	Black, gray, yellow.	Black, golden brown.
2.	Black, gray, yellow; extensive extraction of black.	Golden brown; gray.
3.	Black; red over black; pink.	Black, red, pink.
4.	Black, gray, pink.	Red, pink.

prepared by the Champy-Kull process, all blackened structures may be removed by extraction with turpentine (figs. 11 and 12), and numerous granules (chondriosomes) and droplets are distinctly fuchsinophile (figs. 13 and 14).

Despite all reservations as to specificity of stains, it is difficult to believe that the identity of staining reaction shown by the emulsions of lecithin (figs. 43 and 44) and by the nutritive bodies in the tissues (figs. 11 and 12) following Champy-Kull fixation, together with the exact agreement of both, following the Champy-Kull stain (compare figures 46 and 47 with figures 13 and 14), is a mere coincidence.

Neither is it possible to conclude, after consideration of the established facts concerning the digestion of fats and the known reaction between the oleic fats and osmic acid, that the persistently blackened granules and networks in the Mann-Kopsch preparations of unsaturated fatty acids (figs. 81 to 83) and the persistently blackened structures in the Mann-Kopsch preparations of the tissues (figs. 7, 7a, 8, 8a, and 10) are not of the same chemical nature.

In brief, it is evident that it is only when a mixture of neutral fats, unsaturated fatty acids, lecithin and cephalin is taken, that the fixation and staining reactions of the tissues can be reproduced. If the unsaturated fatty acids are not included in the mixture there will be no persistent blackening after prolonged osmication. If the phospholipins are left out, the mixture will not be fuchsinophile.

NATURE OF THE GOLGI SUBSTANCE

A detailed consideration of the conceptions of the nature of the Golgi apparatus held today would be out of place here. The articles of Owens and Bensley ('29), Bowen ('26, '27), Parat and Painlevé ('24), Nassonov ('26), Guilliermond ('27) and others cover the matter thoroughly.

The considerations offered in this paper apply with equal force to the vacuole (Guilliermond's vacuome) theory proposed by Bensley ('10), and to the original internal reticular apparatus theory of Golgi, if one thinks in terms of substance rather than structure; they apply with equal force to the most complete three dimensional net and to scattered discrete bodies.

It is clear, however, that not all investigators are willing to substitute the word substance for the word structure. Bensley ('29) in a concise critical analysis of the results of metallic impregnations of the Golgi apparatus in which he finds the osmium and the silver methods equally unreliable, says (p. 101), "The reality is the system of spaces with their enveloping membranes revealed by the techniques of Holmgren, Bensley, Parat and Painlevé, and Guilliermond—in other words, the vacuome."

On the other hand Bowen ('26, p. 160) states as a conclusion, "The important thing is that the Golgi apparatus is a substance, the exact modeling of which in the cell is purely a matter of secondary interest."

The authors of the present paper believe that the nature of the evidence that they have secured makes the explanation of Golgi apparatus in terms of substance a logical one.

The histological evidence indicates that processes similar to those occurring in digestion and absorption of fats in the body take place in the growing oöcyte. During digestion, hydrolysis of fats into glycerine and fatty acids occurs. These are absorbed separately and recombined in the cells of the villi of the intestine to form fat, the ester or salt of fatty acid and glycerine. Cramer and Ludford ('25) have shown that in the synthesis of fat, which proceeds in the cells of the intestinal epithelium during fat absorption, the Golgi apparatus is the cell structure mainly concerned.

We know that the particles actively concerned in the vital processes of the cell are of a size beyond the limits of observation with the microscope. Products of their activity become visible only when the granules of product come above this horizon.

The growing oöcyte is not an isolated, independent cell. It is part of the body, whether attached to the ovarian wall or floating in cœlomic liquid. Materials for its growth are supplied to it from the surrounding medium.

The whole picture of accumulation of osmic-blackened substances in the growing egg is that of synthesis of fats. The diffuse cloud, the formation of fine granules, of chains of granules and finally of larger droplets are the visible expression of one series of vital processes—processes that may be correlated by direct observation with the interaction of nucleus and cytosome.

In our material, conformations that can be considered as Golgi *apparatus* are found only as transitional products. They are found in the follicle cells in which fats are being broken down, and in the oöcyte, in which fats are being synthesized. These conformations are not to be found in the mature eggs. In these the persistent blackening is less restricted and the result of secondary osmication is a general blackening of the cytosome.

The results of the study of the tissues, taken by themselves, do not prove the truth of the idea that has been advanced above.

The results of the study of the extracted substances, step by step, from the "total fat" stage to complete separation into fractions show that it is only when fatty acids of the oleic series or of a more highly unsaturated series are present that persistent blackening can be secured. The conclusion that the capacity of the tissues to blacken persistently is due to their presence seems to follow naturally. The ability to blacken persistently after prolonged osmication satisfies the specifications for a Golgi substance, but it is obvious that unsaturated fatty acids can not meet the demands of a general explanation of Golgi substance.

The two sets of experiments, one with lactic acid, the other with reducing sugars, undertaken at this point in the investigation, were intended to reproduce the conditions in muscle and in plants respectively.

The results of these experiments showed conclusively that a considerable variety of substances may reduce osmium tetroxide to a black colloidal solution, from which in the course of hours or days there will be a precipitation or deposit of the dispersed particles.

The fact that osmic acid will blacken fats readily has been known since 1865. Its unreliability as a specific indicator for fat was pointed out by Heidenhain ('88). who recognized the fact that substances other than fat are blackened by osmic acid.

The discovery that brings us closest to the material under consideration in this paper was made by Altmann ('90), who found in testing small quantities of emulsions of oleic, palmitic and stearic acids, and their triglycerides with 2 per cent solution of osmic acid, that only oleic acid and triolein were blackened.

Starke ('95) distinguished in the frog's liver between two kinds of granules, those that blackened during the primary reduction of OsO_4 and those that blackened only after subsequent treatment with alcohol. He confirmed Altmann's observations as to the primary blackening of oleic acid and triolein, and found a secondary blackening of stearin and palmitic acid. Handwerck ('98), upon treating preparations of the saturated fats with hot Marchi's fluid, obtained incomplete blackening that he attributed to the presence of small amounts of oleic acid as an impurity in his fats of higher melting point. Escher ('19) reached similar conclusions.

Bensley ('29) holds that "the blackening of the Golgi apparatus . . . clearly belongs in the secondary phase of the osmic reduction, namely that due to slow reduction by the cytoplasmic proteins, . . ."

The evidence given by the investigations described in the present paper shows that osmium tetroxide is readily decomposed by a number of reducing substances. They are positive in their evidence that the structures in the ovarian tissue of the sea-urchin *Echinometra lucunter*, which may be described as Golgi apparatus, owe their capacity for persistent blackening following prolonged osmication to the presence of fatty acids of the oleic series.

Bowen ('26, page 165) and others have, at least tentatively, advanced the idea that the Golgi substance is chemically composed of two different materials, one lipoid, the other protein in character.

It is never possible to know just what an author means by "lipoid" unless he defines his use of the word. Overton ('01) uses "lipoid" as including the true fats. Some writers use "fats" as including lipoids, others write of "fats and lipoids." Maclean ('27, page 2) suggests that "it would appear best to use the word lipoid to designate the ether-soluble substances extracted from tissues," and adds that "in the literature the word is most frequently used in this sense."

Authors who have suggested lipoid and protein as the probable chemical composition of the Golgi substance have used a term broad enough to include all of the fractions considered in this paper, *i. e.* the true fats—mixtures of unsaturated and saturated fats—cholesterol and its esters, the phospholipins or phosphatids and the galactolipins or cerebrosides. The speculation therefore would seem to be safe, but little can be said for its precision.

The investigations that have been described in this paper enable us, we believe, to define Golgi substance in less general terms than those mentioned above.

So far as the ovarian tissues of the sea urchin *Echinometra lucunter* are concerned, fatty acids of the oleic series, even in a simple aqueous emulsion, in their reaction with osmium tetroxide, meet all of the requirements of a Golgi substance. We can not regard unsaturated fatty acids, however, as the Golgi substance of all tissues.

Our investigations lead us rather to the conclusion that there is no specific Golgi substance characteristic of all cells, as chromatin is characteristic of all nuclei. No other conclusion can be reached if the results of osmic impregnation are used as a criterion.

SUMMARY

The essential feature of this research has been the comparative study of tissues and of substances extracted from tissues, by two standard methods, one of them the Mann-Kopsch method for the demonstration of Golgi substance, the other the Champy-Kull method for the demonstration of chondriosomes.

The aim of the research was the determination of facts concerning the functional significance of Golgi substance and chondriosomes.

The results of the research have been the conviction that neither Golgi bodies nor chondriosomes are structural elements in the cellular architecture, but that both are the chemical products of physiological processes. In other words we have found

no evidence that these are constant structures whose function may be determined, but we have found evidence that not one but several substances may cause the reactions with reagents that we associate with Golgi bodies and chondriosomes.

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Key to Abbreviations

c.—chondriosome
g.e.—germinal epithelium
n.—nucleus
n1.—disintegrating nucleus
n.b.—nutritive body
n.c.—nutritive cell
m.c.l.—muscle and connective tissue layer
m.—muscle fiber
o1.—oögonium
o2.—oöcyte
o3.—mature egg
o.d.—oil droplet

Plate 1

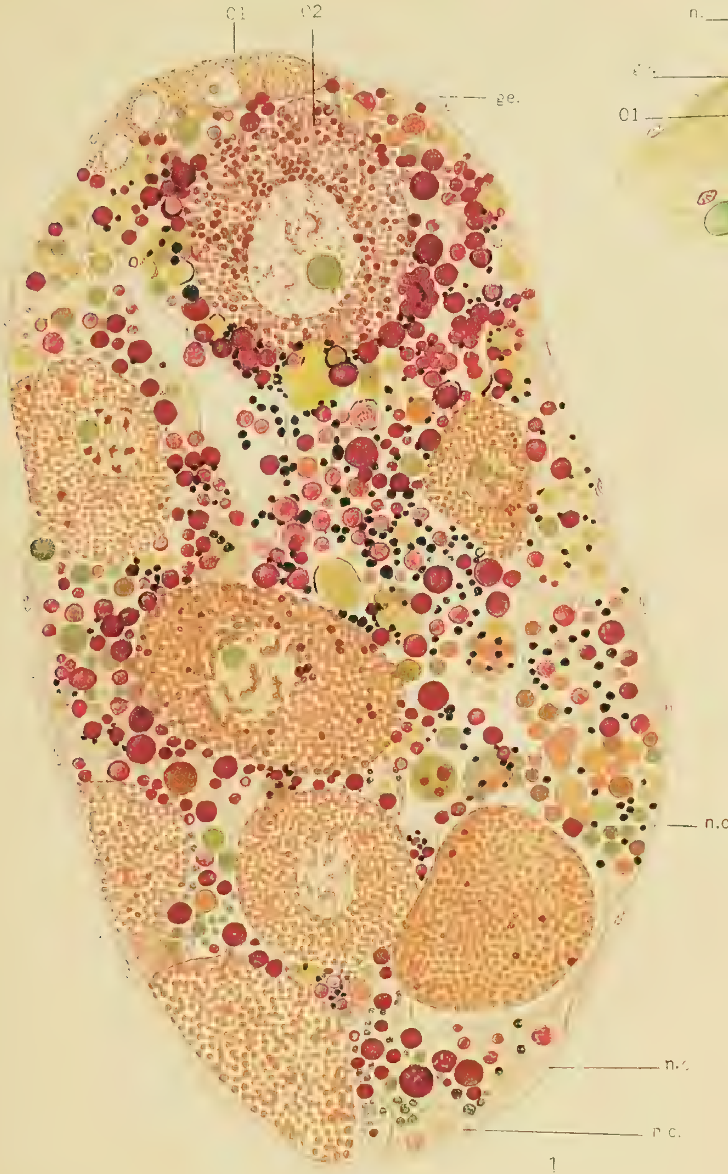
Fig. 1. Longitudinal section through mature ovary, showing oöcytes in wall and disintegrating nutrient cells. Flemming-without-acetic, Champy-Kull stain.

Fig. 2. Portion of wall of ovary in cross-section, showing origin of oöcytes and nutrient cells from epithelium of gonad. Bouin; iron hematoxylin.

Fig. 3. Portion of wall of ovary in cross-section, showing disintegrating nuclei in nutrient cells surrounding oöcytes. Feulgen; light green.

Fig. 4. Section through wall of spent gonad, showing outer epithelial layer and circular muscle layer. Bouin; iron hematoxylin.

Fig. 5. Surface view of epithelial layer of spent gonad, showing cell outlines. Bouin; iron hematoxylin.



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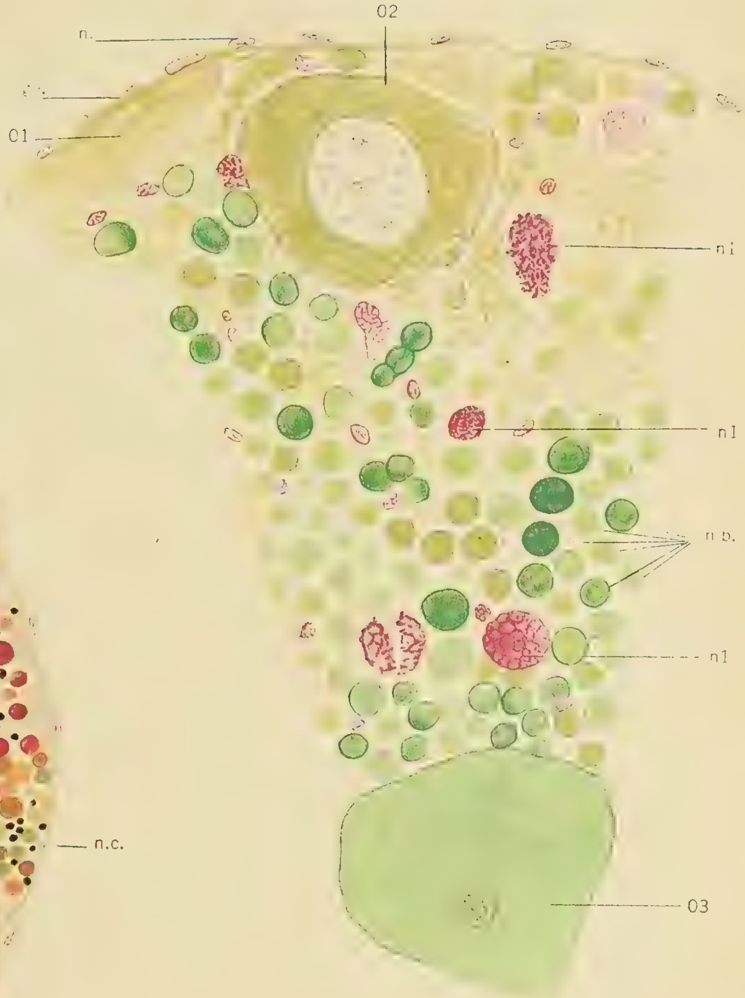

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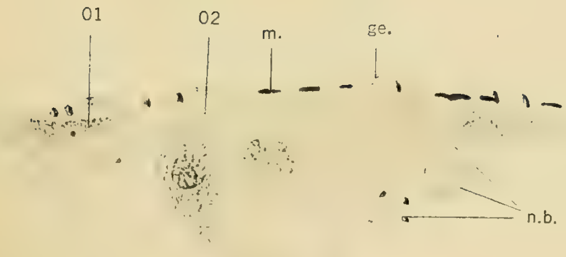

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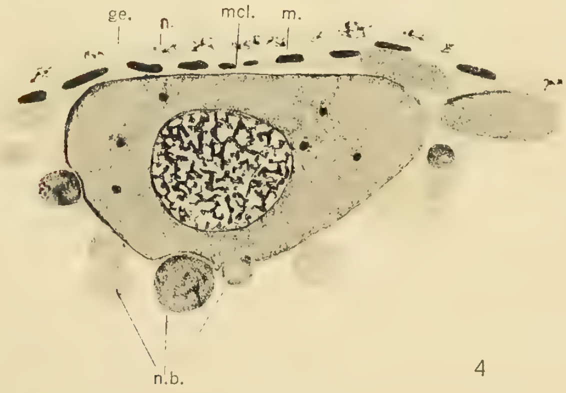

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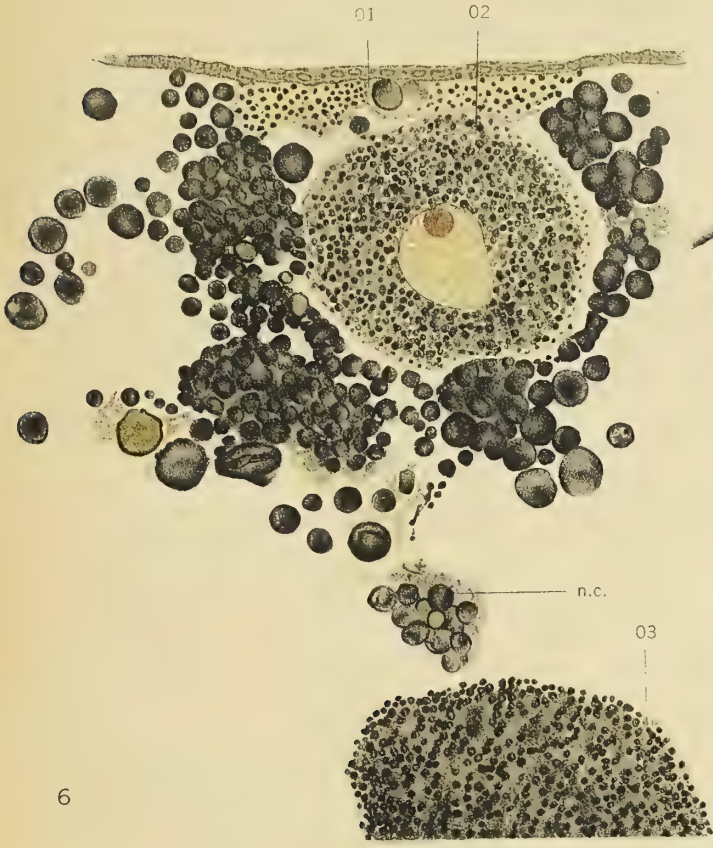
PLATE 2

Figures 6 to 14, Plates 2 and 3 are drawn from sections through mature gonads, showing wall of ovary, developing oöcytes, nutrient cells and mature eggs in the lumen of the ovary.

FIG. 6. Mann-Kopsch, unextracted, unstained.
FIG. 7. Mann-Kopsch, extracted in turpentine 3 minutes, unstained.
FIG. 7a. Detail from figure 7.
FIG. 8. Mann-Kopsch, extracted in turpentine 9 hours, unstained.
FIG. 8a. Detail from figure 8.
FIG. 9. Mann-Kopsch, unextracted, Champy-Kull stain.
FIG. 10. Mann-Kopsch, extracted in turpentine 16 hours, Champy-Kull stain.



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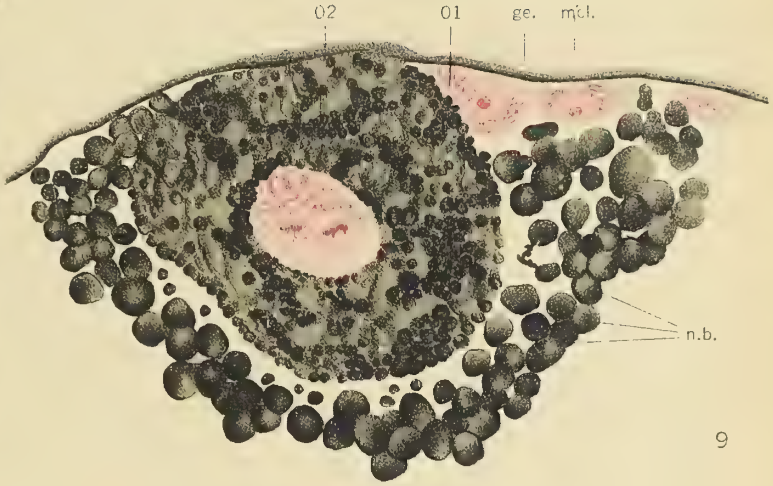

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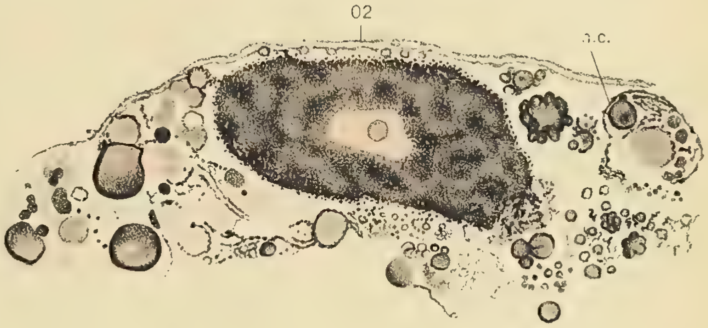

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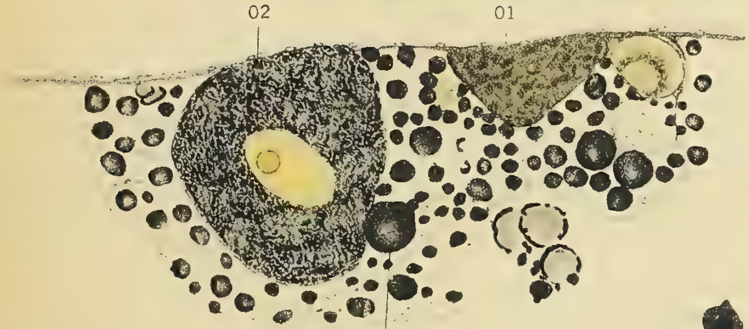

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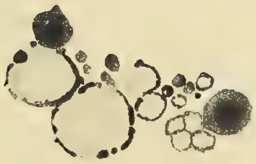

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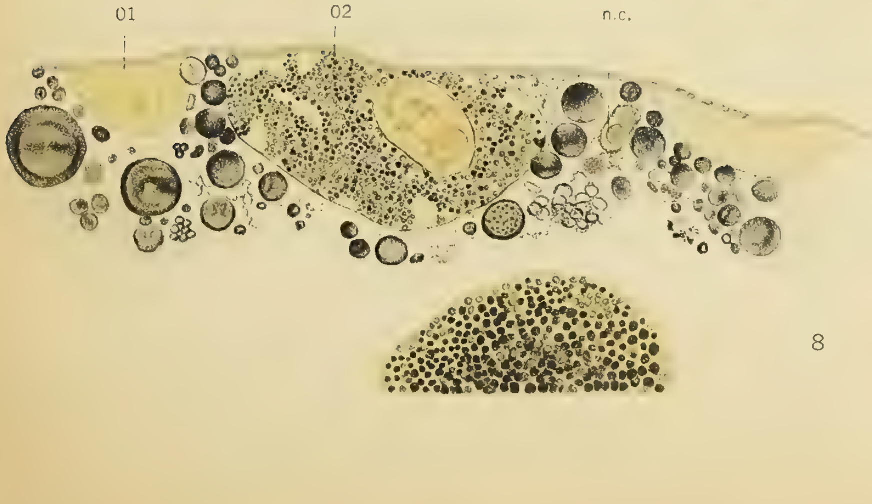

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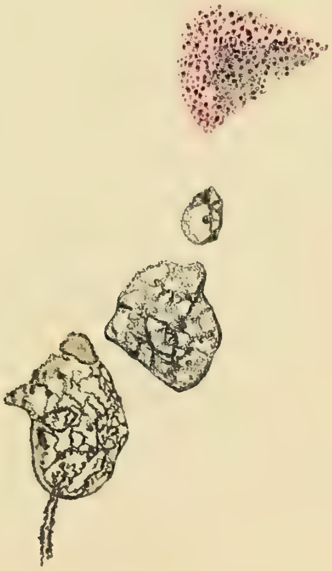

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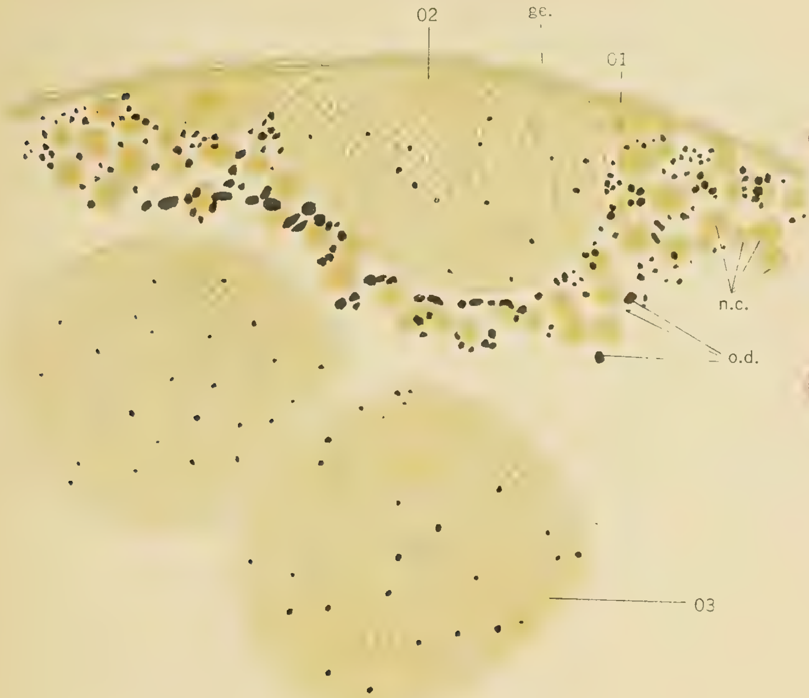
Plate 3

Fig. 11. Champy-Kull, unextracted, unstained.
Fig. 12. Champy-Kull, extracted in turpentine 15 minutes, unstained.
Fig. 13. Champy-Kull, unextracted, Champy-Kull stain.
Fig. 14. Champy-Kull, extracted in turpentine 8 minutes, Champy-Kull stain.



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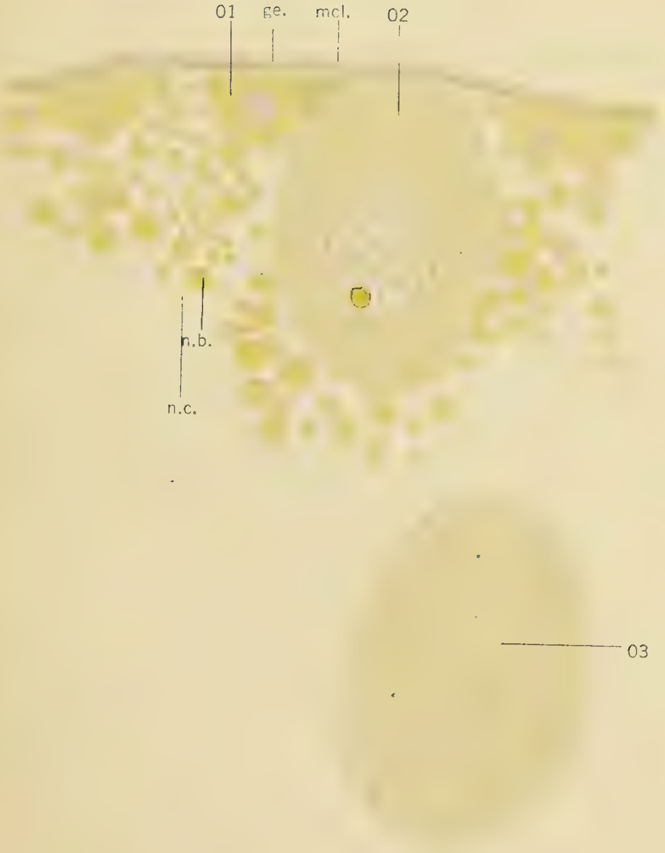

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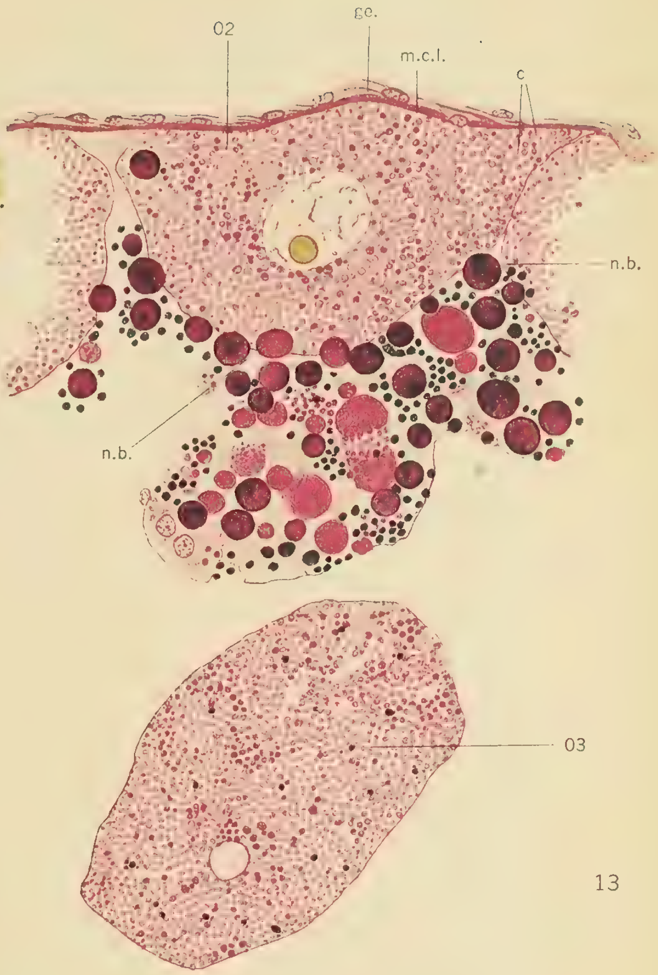

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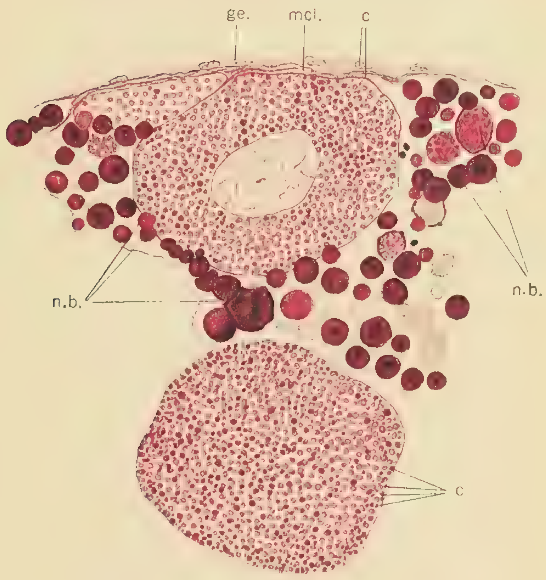

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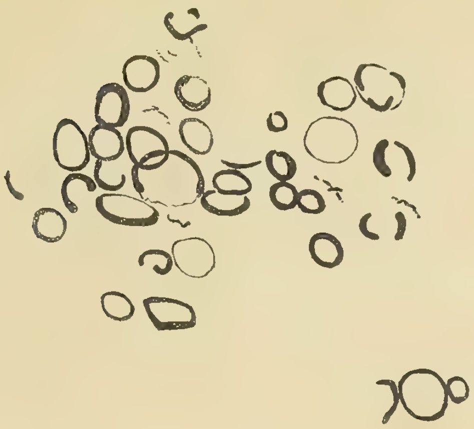
PLATE 4

Total Fats

FIG. 15. Mann-Kopsch, unextracted, unstained.
FIG. 16. Mann-Kopsch, unextracted, unstained.
FIG. 17. Mann-Kopsch, extracted 1 hour 30 minutes in turpentine, unstained.
FIG. 18. Mann-Kopsch, unextracted, Champy-Kull stain.
FIG. 19. Mann-Kopsch, unextracted, Champy-Kull stain.
FIG. 20. Mann-Kopsch, extracted 1 hour 30 minutes in turpentine, Champy-Kull stain.
FIG. 21. Champy-Kull, unextracted, unstained.
FIG. 22. Champy-Kull, extracted 2 hours 30 minutes in turpentine, unstained.
FIG. 23. Champy-Kull, extracted 2 hours 30 minutes in turpentine, unstained.
FIG. 24. Champy-Kull, unextracted, Champy-Kull stain.
FIG. 25. Champy-Kull, unextracted, Champy-Kull stain.
FIG. 26. Champy-Kull, $KMnO_4$, 15 minutes, Champy-Kull stain.
FIG. 27. Champy-Kull, $KMnO_4$, Champy-Kull stain.
FIG. 28. Champy-Kull, extracted 20 minutes in turpentine, Champy-Kull stain.
FIG. 29. Champy-Kull, extracted 1 hour 15 minutes in turpentine, Champy-Kull stain.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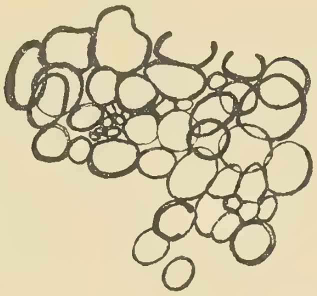
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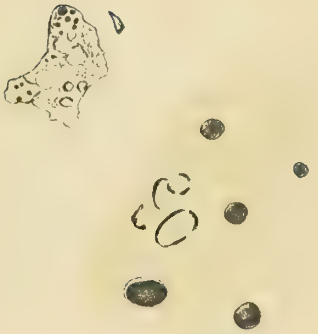
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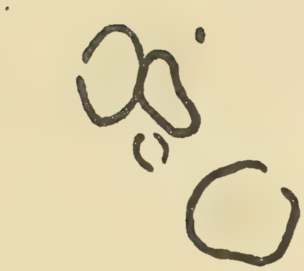
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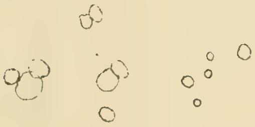
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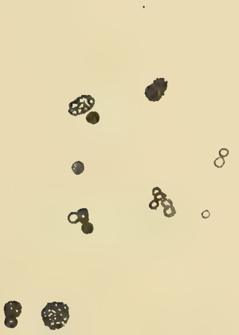
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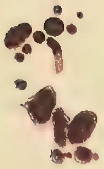
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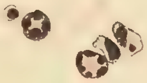
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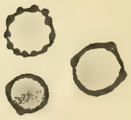
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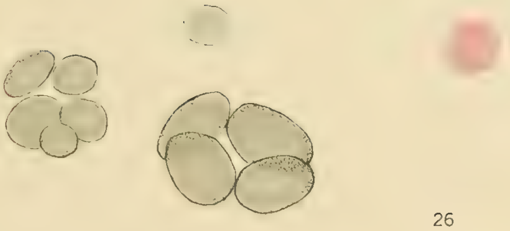
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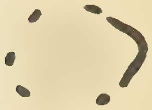
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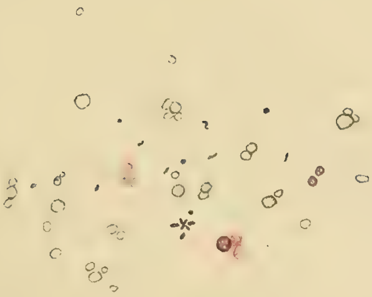
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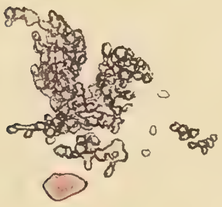
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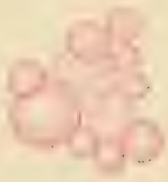
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PLATE 5

Solution of cephalin, figures 30 to 37; emulsion of lecithin, figures 38 to 47

FIG. 30. Mann-Kopsch, unextracted, unstained.
FIG. 31. Mann-Kopsch, extracted 3 minutes, unstained.
FIG. 32 Mann-Kopsch, unextracted, stained.
FIG. 33. Mann-Kopsch, extracted, stained.
FIG. 34. Champy-Kull, unextracted, unstained.
FIG. 35. Champy-Kull, extracted, stained.
FIG. 36. Aqueous solution of cephalin from sheep brains, fixed in osmic vapor 4 hours. 4D, camera.
FIG. 37. Aqueous solution of cephalin from sheep brains, fixed in osmic vapor 4 hours. 4D, camera.
FIG. 38. Mann-Kopsch, collodion, unextracted, unstained, 8:2mm.
FIG. 39. Mann-Kopsch, collodion, extracted 15 minutes in turpentine, unstained. 8:2mm.
FIG. 40. Mann-Kopsch, extracted 3 days in turpentine, unstained. 8:2mm.
FIG. 41. Mann-Kopsch, unextracted, stained. 8:2mm.
FIG. 42. Mann-Kopsch, collodion, extracted 1 hour in turpentine, stained. 8:2mm.
FIG. 43. Champy-Kull, unextracted, unstained.
FIG. 44. Champy-Kull, unextracted, unstained.
FIG. 45. Champy-Kull, extracted 30 minutes in turpentine, unstained.
FIG. 46. Champy-Kull, unextracted, stained.
FIG. 47. Champy-Kull, extracted 1 hour in turpentine, stained.

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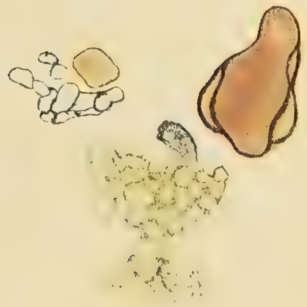
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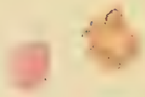
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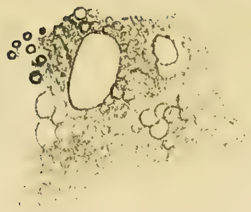
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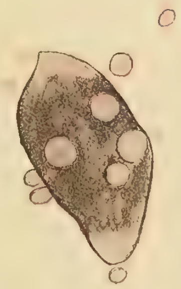

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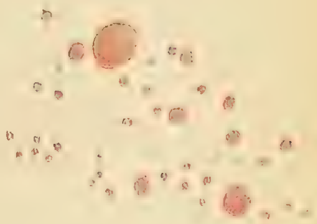
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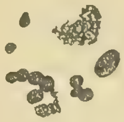
PLATE 6

Emulsion of commercial lecithin, figures 48 to 58; emulsion of commercial cholesterol, figures 59 to 61; cholesterol extracted from *Echinometra,* figures 62 and 63.

FIG. 48. Mann-Kopsch, unextracted, unstained.
FIG. 49. Mann-Kopsch, extracted in turpentine 10 minutes, unstained.
FIG. 50. Mann-Kopsch, extracted in turpentine 30 minutes, unstained.
FIG. 51. Mann-Kopsch, unextracted, stained.
FIG. 52. Mann-Kopsch, extracted in turpentine 15 minutes, stained.
FIG. 53. Mann-Kopsch, extracted in turpentine 75 minutes, stained.
FIG. 54. Champy-Kull, unextracted, unstained.
FIG. 55. Champy-Kull, extracted in turpentine 10 minutes, unstained.
FIG. 56. Champy-Kull, extracted in turpentine 30 minutes, unstained.
FIG. 57. Champy-Kull, unextracted, stained.
FIG. 58. Champy-Kull, extracted in turpentine 15 minutes, stained.
FIG. 59. Mann-Kopsch, unextracted, unstained.
FIG. 60. Mann-Kopsch, extracted in turpentine 24 hours, unstained.
FIG. 61. Mann-Kopsch, unextracted, unstained.
FIG. 62. *Echinometra* cholesterol crystals from alcoholic solution.
FIG. 63. *Echinometra* cholesterol crystals from ethereal solution.



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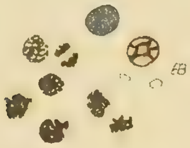

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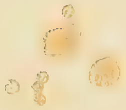

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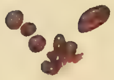

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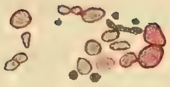

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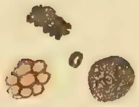

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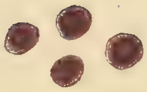

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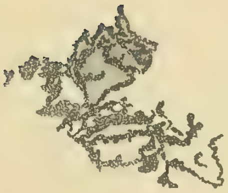

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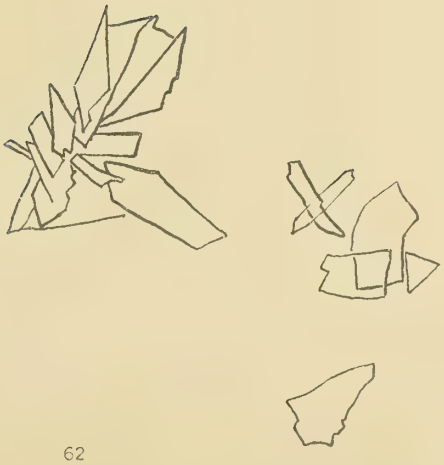

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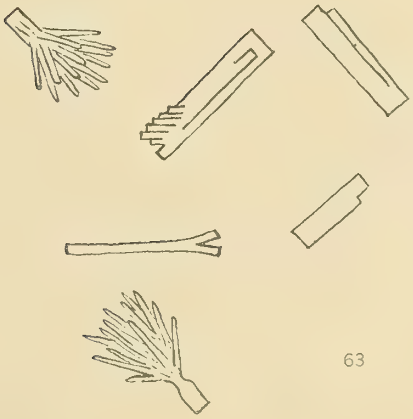

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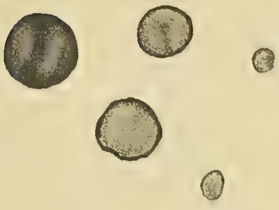
PLATE 7

Fat and cholesterol fraction, figures 64 to 78; saturated fatty acids, figures 79 to 80; unsaturated fatty acids, figures 81 to 86; chloroform and aqueous solution of Nile blue sulphate, figure 87.

FIG. 64. Mann-Kopsch, unextracted, unstained.
FIG. 65. Mann-Kopsch, short extraction, unstained.
FIG. 66. Mann-Kopsch, extracted 24 hours in turpentine, unstained.
FIG. 67. Mann-Kopsch, unextracted, stained.
FIG. 68. Champy-Kull, unextracted, unstained.
FIG. 69. Champy-Kull, unextracted, unstained.
FIG. 70. Champy-Kull, extracted 23 minutes in turpentine, unstained.
FIG. 71. Champy-Kull, extracted 48 hours in turpentine, stained.
FIG. 72. Mann-Kopsch fixation of alcoholic extract of supernatant layer of ethereal solution, extracted 20 minutes in turpentine.
FIG. 73. Mann-Kopsch fixation of concentrated ethereal solution of fat and cholesterol fraction, extracted in turpentine 45 minutes, unstained.
FIG. 74. Mann-Kopsch fixation of aqueous emulsion of concentrated ethereal solution of fat and cholesterol fraction, extracted 24 hours in turpentine, unstained.
FIG. 75. Mann-Kopsch fixation of aqueous emulsion of concentrated ethereal solution of fat and cholesterol fraction, extracted 24 hours in turpentine, unstained.
FIG. 76. Mann-Kopsch fixation of aqueous emulsion of concentrated ethereal solution of fat and cholesterol fraction, $KMnO_4$ 30 seconds, stained.
FIG. 77. Mann-Kopsch fixation of a drop of concentrated ethereal solution of fat and cholesterol fraction, extracted 24 hours in turpentine, unstained.
FIG. 78. Champy-Kull fixation of aqueous emulsion of alcoholic extract of concentrated ethereal solution of fat and cholesterol fraction, unextracted, stained.
FIG. 79. Mann-Kopsch fixation of aqueous emulsion of chloroform solution of saturated fatty acids, unextracted, stained.
FIG. 80. Mann-Kopsch fixation of aqueous emulsion of ethereal solution of saturated fatty acids, extracted 40 minutes in turpentine, unstained.
FIG. 81. Mann-Kopsch fixation of aqueous emulsion of alcohol-ether solution of unsaturated fatty acids, extracted 10 minutes in turpentine, unstained.
FIG. 82. Mann-Kopsch fixation of alcohol-ether-water emulsion of ethereal solution of unsaturated fatty acids, extracted 30 minutes in turpentine, unstained.
FIG. 83. Mann-Kopsch fixation of aqueous emulsion of alcohol-ether solution of unsaturated fatty acids, extracted 24 hours in turpentine, unstained.
FIG. 84. Champy-Kull fixation of aqueous emulsion of alcohol-ether solution of unsaturated fatty acids, unextracted, unstained.
FIG. 85. Champy-Kull fixation of aqueous emulsion of alcohol-ether solution of unsaturated fatty acids, extracted 5 minutes in turpentine, unstained.
FIG. 86. Flemming-without-acetic fixation of aqueous emulsion of ethereal solution of unsaturated fatty acids, treated with permanganate and oxalic, stained.
FIG. 87. Droplets of chloroform in a chloroform-water mixture, stained with Nile blue sulphate.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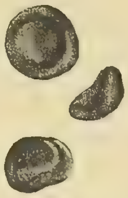
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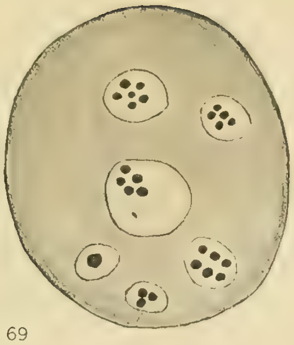
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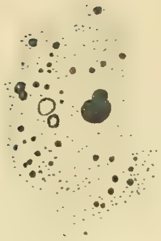
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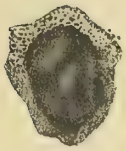
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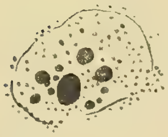
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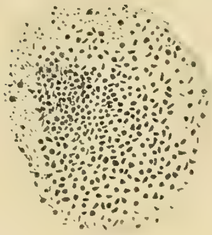
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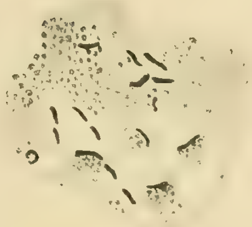
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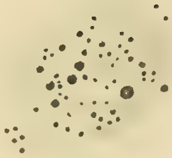
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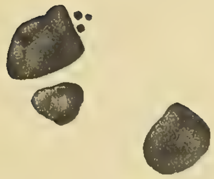
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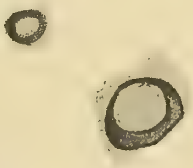
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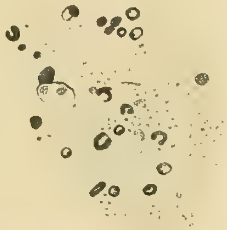
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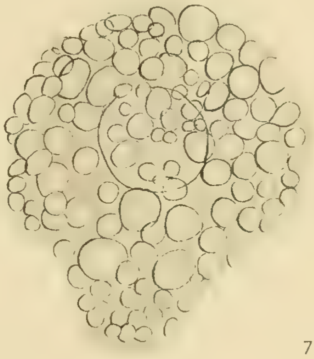
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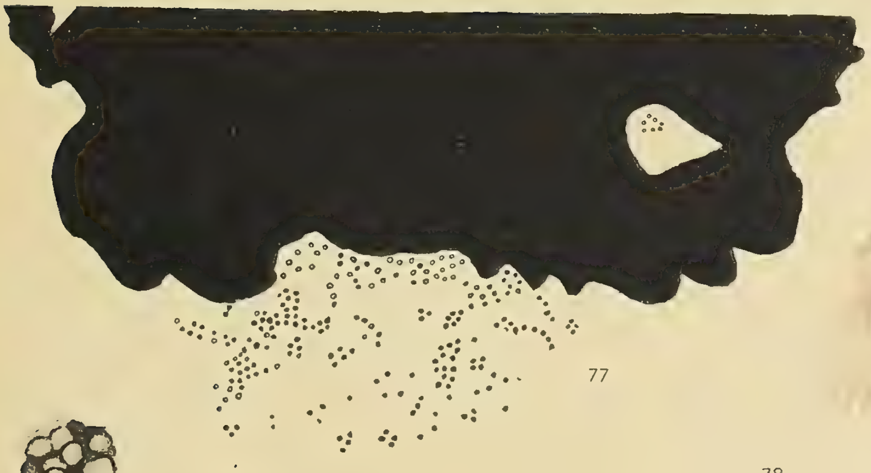
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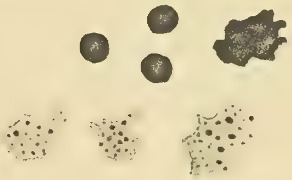
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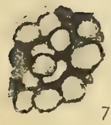
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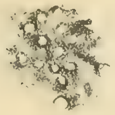
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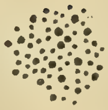
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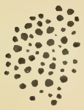
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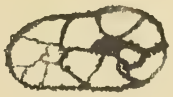
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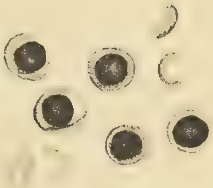
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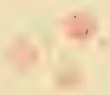
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SECTION II

A BIOCHEMICAL STUDY OF THE EGGS AND OVARIES OF THE SEA-URCHIN ECHINOMETRA LUCUNTER

By Dorothea E. Smith

DETERMINATION OF THE LIPIDES AND OF GLYCOGEN

The primary object of this investigation was to isolate the various lipides present in this tissue in order that their staining reactions might be compared with those of the cytoplasmic inclusions, and also to ascertain by analysis the amounts of lipides and of glycogen present. The determination of the unsaturation was made because it seemed possible that there was a relation between the unsaturation and the reaction to stains.

The determination of the iodine number affords a few observations on the unsaturation of the lipides from the tissue of an invertebrate to compare with the many studies on the fats of warm-blooded animals, especially mammals (Bloor, '26, '27).

The published analyses of eggs are few in number and indicate great variation in the relative amounts of the substances furnishing energy for development in the species studied. Needham has reviewed the work done in this field ('25). The ovaries, or eggs alone, of *Echinometra lucunter* contain glycogen in amounts which are readily demonstrated and quantitatively determined. This has not been reported to be the case in the egg of other species of sea-urchins. Apparently it has been found in small amounts in one species, *Arbacia punctulata* (Perlzweig and Barron, '28) and not at all in another, *Strongylocentrotus lividus* (Meyerhof, '11).

The *Echinometra* tissue was collected at Tortugas, weighed and placed in redistilled 97.5 per cent alcohol. After about one hour of dehydration, the alcoholic supernatant, which had already extracted a considerable amount of fat and pigment, was poured off and carefully preserved, and fresh alcohol placed in the original bottle. About 300 grams of tissue were taken for each of the three analyses. Glass-stoppered bottles were used to transport the tissue in alcohol.

The analysis was carried out with the usual precautions against oxidation. Materials were kept in the ice-box, ethereal solutions dried with anhydrous sodium sulfate, under an atmosphere of CO_2, or else the substances were dried and kept in a vacuum desiccator over P_2O_5.

The dehydration and the supernatant alcohol were filtered through a double plaited filter and evaporated under reduced pressure. The tissue was placed on the same filter, 100 to 150 grams at a time, and extracted with 96 per cent alcohol in a percolator according to the method of Bloor ('16) and of Sperry ('26). Extraction had to be much longer continued, with a change of alcohol every two hours, until orange-colored pigment and fat were no longer removed. The dehydrated tissue was then quickly dried, pulverized in a mortar and replaced on the filter as nearly quantitatively as possible and further extracted with alcohol and ether.

The alcoholic (and ether) extracts from each analysis were evaporated under reduced pressure, at 32° to 37° C., and the residue, together with that from the dehydration and supernatant alcohol, dissolved in ether and made up to 500 c.c. in a volumetric flask. From this aliquot parts were removed from time to time for the various determinations. The flasks were kept in the ice-box under CO_2 and, if not worked up at once, were weighed, so that any ether lost by evaporation could be replaced.

In the case of the dehydration alcohol, there was some water left after the alcohol had been removed by evaporation. This was shaken out with ether and the ether used in dissolving the residues from the alcoholic extracts. The water was evaporated off on the steam bath and the weight of the alcohol-soluble ether-insoluble substances found.

The iodine number was determined by the method of Wijs on the residue of a 5 c.c. aliquot of the 500 c.c. ethereal solution containing the total fat extracted from the tissue in the course of each analysis. The residue of a similar 5 c.c. aliquot was dried in a vacuum desiccator over P_2O_5 to constant weight as a basis for calculating the iodine number and also the total amount of fat extracted. Two or three such aliquots were always weighed.

The ether-soluble phosphorus was determined by the method of Fiske and Subbarow ('25) after digestion of the residue of an aliquot with a mixture of equal parts of concentrated H_2SO_4 and HNO_3, as suggested by Robertson and Wasteneys for similar material ('13).

Cholesterol was determined colorimetrically on 1 or 2 c.c. aliquots by the method of Bloor ('16). It was observed that the deep green color of the unknown developed with unusual rapidity, almost at once, but no explanation is offered.

The previous year glycogen had been found to be present. Desiccated tissue that had been preserved in 80 or 95 per cent ethyl alcohol was digested with 30 per cent KOH on the water bath for several hours. One volume of 96 per cent ethyl alcohol was added to precipitate the glycogen. A solution of this showed the characteristic "port wine" color on adding iodine-potassium-iodide solution. After hydrolysis with hydrochloric acid, an orange-colored precipitate came down with Benedict's reagent, but not before hydrolysis. Similarly, after the action of ptyalin a reducing sugar could be demonstrated, but not before. For the estimation of glycogen the well-known Pflüger ('09) method was employed with some necessary modifications. The alcohol precipitation was once repeated, and the suggestion of Lovatt Evans ('26) that the volume of solution from which the glycogen is to be precipitated should not exceed 1 c.c. per milligram of glycogen expected was carefully followed (page 367). After hydrolysis, the glucose was determined by the method of Lehman-Maquenne (Griesbach und Strassner, '13). Working with the first lot of gonad tissue from Tortugas it was found that the glycogen made up 0.69 per cent of the wet weight. A similar analysis of 0.7686 gram of *eggs only*, carefully dried in a vacuum desiccator over P_2O_5, was made. The glucose found amounted to 19.31 mgs., or 2.33 per cent glycogen in the dried matter of the eggs. Analyses of gonad tissue obtained more recently gave the following results:

From 0.4775 gram tissue, after extraction with alcohol and drying, 63.98 mgs. glucose, or 12.42 per cent.

From 0.4945 gram tissue, after extraction with alcohol and drying, 67.85 mgs. glucose, or 12.72 per cent.

Pflüger's conversion figure, 0.927 (times glucose found) was used in calculating the percentage of glycogen.

Summary of analytical results

	Analysis I	Analysis II	Analysis III
Weight of fresh tissue at Tortugas	304.8 gms.	316.6 gms.	304.4 gms.
Residue, after extraction and drying	50.026 gms.	48.238 gms.	46.862 gms.
Extractives, alcohol-soluble, ether-insoluble	11.454 gms.	14.359 gms.	14.115 gms.
Weight of fat dissolved in 5 c.c. of ethereal solution of total fat extracted	0.2750 gm. 0.2745 gm.	0.2759 gm. 0.2749 gm.	0.2727 gm. 0.2732 gm. 0.2734 gm.
Average	0.2747 gm.	0.2754 gm.	0.2731 gm.
Total fat extracted (volume of ethereal solution 500 c.c.)	27.47 gms.	27.54 gms.	27.31 gms.
Residue, extracted and dried, p. ct. of fresh tissue	16.42	15.24	15.39
Extractives, alcohol-soluble, ether-insoluble, p. ct. tissue	3.76	4.54	4.64
Lipids extracted, p. ct. of the fresh tissue	9.01	8.70	8.97
Total dried solids, p. ct. of fresh tissue	29.19	28.48	29.00
Glycogen, p. ct. of the dried extracted residue	12.42 12.72		
Iodine number, determined on total fat dissolved in 5 c.c. of ethereal solution	84.5 84.4	85.8 85.8	82.6 82.4
Ether-soluble phosphorus in 1 c.c. of ethereal extract of total fats	0.440 mg.	0.446 mg.	0.441 mg.
Total ether-soluble phosphorus extracted	0.220 gm.	0.223 gm.	0.220 gm.
Ether-soluble phosphorus, p. ct. of fresh tissue	0.072	0.070	0.072
Cholesterol in 1 c.c. of ethereal extract of total fats	3.61 mgs.	4.20 mgs. 4.15 mgs.	5.88 mgs. 5.85 mgs.
Average	3.61 mgs.	4.17 mgs.	5.87 mgs.
Total cholesterol extracted	1.805 gms.	2.088 gms.	2.934 gms.
Cholesterol extracted, p. ct. of fresh tissue	0.592	0.66	0.96
Calculated amount of phosphatides, on basis of one molecule of lecithin to one of cephalin, total extracted	5.486 gms.	5.561 gms.	5.486 gms.
Phosphatides extracted, p. ct. of fresh tissue	1.80	1.76	1.80
Neutral fat and fatty acid, exclusive of phosphatides and cholesterol (estimated by difference, not determined), total extracted	20.18 gms.	19.89 gms.	18.89 gms.
Neutral fat and fatty acid, exclusive of phosphatides and cholesterol estimated p. ct. of fresh tissue	6.62	6.28	6.21

ISOLATION OF THE PRINCIPAL LIPIDES AND DETERMINATION OF THE UNSATURATION

What was left of the ethereal solution in the three volumetric flasks after the various determinations had been made was put together and evaporated under reduced pressure to a volume of about 300 c.c. It was then placed in the refrigerator over night. The fraction that settled out—the "cerebroside fraction"—amounted to 7.6 grams. After 3 hours' hydrolysis with 16 c.c. of 3 per cent H_2SO_4, 8 mgs. of glucose were obtained from 0.9932 gram of the material.

We wish at this point to express our gratitude to Dr. P. A. Levene for examining this fraction for us. He reported that it "apparently consisted of crude white matter, which is a mixture of cerebrosides, sphyngomyelin and other things." The analyses of two fractions into which it was separated, for which we are indebted to him, are as follows: P 1.51, N 2.37, C 59.34, H 9.98 per cent (more insoluble fraction). The material contained 16 per cent mineral residue. The figures were recalculated on the basis of the ash-free material. The second fraction of the same material contained P 1.89, N 3.09, C 59.45, H 9.39, and 7.46 per cent mineral residue.

The ethereal solution left after the "cerebroside fraction" had settled out was poured into 300 c.c. of 98.5 per cent ethyl alcohol, redistilled, and warmed to 60°C., according to the method of Levene and Rolf ('27 B). There was a small amount of gummy precipitate and a little fine white powder came down. This precipitate, representing the "cephalin fraction," was extracted with warm alcohol to remove the fat and purified by dissolving in ether four times and reprecipitating as before. It had apparently oxidized to a considerable extent.

0.1063 gram of substance absorbed 0.0494 gram iodine by the Wijs method. The iodine number, in two titrations, was found to be 46.5.

Another "cephalin fraction" was prepared in 1929, from about 300 grams of gonad tissue. Since this fraction was small in amount and a large proportion of it insoluble in ether, attempts at further purification had to be given up. We are therefore particularly grateful to Dr. Levene for having made for us an analysis of the small portion available.

4.100 mg. substance gave: 8.900 mg. CO_2 and 3.350 mg. H_2O and 0.340 mg. ash: C 59.19, H 9.14, ash 8.29 per cent. Calculated ash-free: C 62.96, H 9.72, N 1.66, P 1.09, and amino-nitrogen 0.52 per cent.

To the filtrate, after removal of the "cephalin fraction" was added saturated alcoholic cadmium chloride solution (Levene and Rolf, '27). After standing 24 hours in the cold, the cream-colored precipitate consisting of the cadmium chloride salt of lecithin was filtered off, washed in very dry ether and centrifuged. The washing and centrifuging was repeated until the supernatant was colorless (about 5 times). The cadmium chloride salt of lecithin was then suspended in chloroform dried over sodium. The cadmium was precipitated by adding dry methyl alcohol into which had been passed dry ammonia gas. After centrifuging, the supernatant was evaporated under reduced pressure and the residue taken up in a little dry ether. This solution was then poured into acetone which had been dried over fused $CaCl_2$ and redistilled. The precipitation with acetone was once repeated, but there was not enough material to purify further according to the method of Levene and Rolf ('27). The lecithin was dried and kept in a vacuum desiccator over P_2O_5. The iodine number was determined.

0.6514 gram substance absorbed 0.6998 gram of iodine according to the method of Wijs. The iodine number was found to be 107.5.

The filtrate from the cadmium chloride salt of lecithin was treated with dry ammonia in dry methyl alcohol to remove the excess cadmium chloride. The solution was evaporated under reduced pressure until the odor of ammonia could no longer be detected. The iodine number was determined.

0.2130 gram substance absorbed 0.1935 gram iodine by the Wijs method. The iodine number was found to be 90.9.[1]

The rest of the material, 29.9 grams of neutral fat, fatty acids and cholesterol, was refluxed 2 hours on the water-bath with 12 grams of KOH in 60 c.c. of 96 per cent ethyl alcohol. It was washed repeatedly with ether (about 13 times), using the proportions recommended by Boehmer (as quoted in Leathes and Raper, '25) to avoid emulsification and the cholesterol so obtained again saponified with 2 grams of KOH.

Since Page ('23) had reported a new sterol from the star-fish, the cholesterol isolated was carefully studied. It gave the usual color reactions and showed the same crystalline form as ordinary cholesterol. After three recrystallizations, it melted quite sharply at 143° C. After again crystallizing, it melted sharply at 144° C. (uncorrected).

The solution containing the soaps was acidified and the fatty acids extracted with ether and washed until free from mineral acid. The solution was dried over fused Na_2SO_4 and evaporated to dryness under reduced pressure. The solid cake of fatty acids was dissolved in warm methyl alcohol (redistilled) and treated with lead acetate in a small amount of water and a little ammonia in methyl alcohol. The reaction mixture was chilled to $-5°$ C. with ice and salt, and the separated lead salts of the saturated and unsaturated fatty acids obtained by centrifuging (Levene and Simms, '21). They were washed repeatedly in the centrifuge tubes with boiling ether until the supernatant was colorless.

The sediment, now perfectly white, consisting of the lead salts of the saturated fatty acids, and the orange-colored mixed washings consisting of the lead salts of the unsaturated fatty acids, were each decomposed in a separatory funnel with *dilute* HNO_3 (Twitchell, '21). The fatty acids were extracted with ether and the ethereal solution washed with distilled water, and a solution of Na_2SO_4 when made necessary by emulsion formation, and dried over Na_2SO_4. 7.75 grams of unsaturated fatty acids were obtained. The iodine number was determined.

0.3623 gram substance absorbed 0.3635 and 0.3696 gram of iodine in two titrations. The iodine number was found to be 100.32 and 99.25, respectively. Average, 99.78. This indicates the presence of a more highly unsaturated fatty acid than oleic acid.

About 8 grams of saturated fatty acids were obtained. The melting point was 50° to 52° C.

SUMMARY

Analytical figures for the percentage of lipides and of glycogen are given. The amount of the latter, 12.42 per cent, and 12.72 per cent of the dried extracted tissue is high. In addition, the presence of cerebrosides and sphyngomyelin are indicated. The lipide composition of this tissue seems to be as complex as that of any.

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[1] A preparation of neutral fat and cholesterol only was made for a study of staining reactions as follows. A solution containing fat, fatty acids and cholesterol only in chloroform was titrated with alcoholic NaOH, using phenolphthalein as external indicator. The jelly-like mass of soap was removed by centrifuging, and the chloroform solution was washed repeatedly with distilled water, using about six litres. The chloroform was then evaporated to dryness under reduced pressure, and the last traces of chloroform removed by dissolving the residue in dry ether and again evaporating to dryness. The neutral fat and cholesterol was then dried in a vacuum desiccator over P_2O_5.

To obtain neutral fat only, the cholesterol was precipitated from the preparation which had been freed from fatty acids as described above with 1 per cent alcoholic digitonin, kindly given to us by Dr. W. A. Jacobs. The precipitate was removed by centrifuging. Excess digitonin was removed by washing a solution of the neutral fat in petroleum ether with distilled water. The neutral fat was dried in a vacuum desiccator over P_2O_5 after evaporating off the petroleum ether.

The unsaturation of several preparations used in the study of staining reactions was determined, to find out if there was any correlation between unsaturation and staining with osmic acid (see Section I). On the whole the lipides are probably more unsaturated than similar preparations from mammalian tissues.

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II

OBSERVATIONS ON THE FORMATION OF THE EGG OF ECHINOMETRA LUCUNTER

Ruth A. Miller and Helen B. Smith

With six plates

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OBSERVATIONS ON THE FORMATION OF THE EGG OF ECHINOMETRA LUCUNTER

INTRODUCTION

Our knowledge of the formation and growth of the sea-urchin egg is founded on the observations of Hamann ('87) whose work forms the basis of the description given by Korschelt and Heider (('02). The work done by more recent authors has not added materially either to our knowledge of the structure of the gonad or to our information concerning the processes of vitellogenesis.

The study of the ovaries of *Echinometra* was undertaken in the hope that by the use of some of the newer cytological methods, it might be possible to extend our knowledge of the processes of oögenesis, particularly of those concerned in the formation of yolk in the echinoid egg.

This paper presents the observations that have been made, and the conclusions that have been drawn.

We take this opportunity of expressing our indebtedness to Doctor David H. Tennent for his help and criticism. Our work was undertaken at his suggestion. The material was collected and fixed by him at the Tortugas Laboratory of the Carnegie Institution of Washington late in July 1926, as part of that to be used in a microchemical study of biological material. At the time that the material was collected, *Echinometra* was about to enter its breeding season. The ovaries were therefore in an advanced stage of development, and hence the observations on oögenesis are limited to a smaller number of eggs than if the material had been collected earlier. The ovaries are of the sac-like type, opening to the exterior through a single duct, with finger-like projections containing eggs and accessory cells.

MATERIAL AND METHODS

In the preparation of the material, four methods of fixation were used: Flemming-without-acetic acid,Bouin, Bensley-Altmann and Regaud. Of these, Flemming-without-acetic and Bouin were the most satisfactory. The Flemming-without-acetic material was stained by the Champy-Kull acid fuchsin, toluidin blue or thionin, and aurantia process, and also by Benda's crystal violet method. The Bouin material was stained by Heidenhain's iron hematoxylin method and with iron hematoxylin in combination with eosin. The Champy-Kull method gave very striking results. The eggs stained brilliantly and the formed components of the tissues showed many varieties of color. The crystal violet method, though less spectacular in effect, was more useful in the differentiation of granular contents. The iron hematoxylin method was used in the study of chromosomes, and combined with eosin emphasized the contrast between cytoplasm and nuclear structures.

OBSERVATIONS ON OÖGENESIS

Sections showed that the wall of the ovary was composed of a single layer of cells surrounded on the outside by a muscle layer.[1] Small, undifferentiated cells

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[1] Gardiner, Smith and Tennent ('30), after the study of material collected later, have shown that there is an epithelial layer outside of the muscle layer.

(Plate 1, fig. 1), with granular cytoplasm, lay along the inner side of the wall close to the muscle layer. These cells were very uniform in appearance.

FORMATION OF THE FOLLICLE CELLS

In the wall of the ovary there were also cells resembling the undifferentiated cells in content, but which were larger. These cells were of two types: those in which nuclear material could not be seen and those in which it was visible. In the first type of cell the cytoplasm seemed to be in a process of disintegration (Plate 1, fig. 2) and appeared to be forming masses which rounded up into granular bodies (Plate 1, fig. 3). Owing to differences in staining capacity, many of the spheres appeared homogeneous, but in reality they were simply more densely granular than the others. The granules of the fully formed bodies were on the whole coarser and less dense than those of the cytoplasm from which they were formed. In the second type of cell the nuclear material seemed to be fragmented and distributed throughout the cytoplasm, giving the cell the appearance of a syncytium (Plate 2, figs. 1 and 2).

The bodies formed during this process of disintegration may be termed "nutritive spheres," "deutoplasmic" or "yolk-forming" bodies, inasmuch as they are concerned with the formation of deutoplasm or yolk. It is not intended to imply that these bodies are directly transformed to yolk. They are instead simply spherules of nutritive material.

After the complete disintegration of the enlarged cell, some of the resulting bodies, containing both large and small granules, have the appearance of cytoplasm (Plate 2, fig. 3, d_1), some are composed of both cytoplasmic and nuclear material (d_2), while others are clear and non-granular (d_3). Each kind of body gave the staining reaction characteristic of the material of which it was formed, thus furnishing a means of identifying its composition.

The manner of origin of these granules has not been determined. A finely granular condition was succeeded by a coarsely granular one. As the staining reaction remained the same throughout, it is possible that the coarse granules are an aggregation of the more numerous fine ones (Plate 1, figs. 2 and 3).

With the Champy-Kull stain, the yolk-forming bodies were clear and showed no granular content. With the Benda stain, the granules were easily distinguishable, the large granules staining more deeply than the small ones. Most of the yolk-forming bodies were granular following the use of the Benda method, although some retained their homogeneous appearance.

These bodies and granules might be described as chondriosomes and their phospho-lipoid nature assumed. It seemed desirable, however, to make a specific test of the nature of these structures, and Gardiner's ('27) adaptation of Bell and Doisy's technique was used for this purpose. By means of the ammonium molybdate test (Gardiner '27, p. 645) the bodies and granules were shown to contain organic phosphorus in the form of orthophosphate. From this it may be concluded that the yolk-forming bodies are composed of phospho-lipins suspended in a homogeneous lipoidal medium. In biochemical studies on *Echinometra* eggs and ovaries now being made in this laboratory this material is shown to contain a high lecithin content. In all probability the phospho-lipoid granules are lecithin.

Disintegration continued in the large cells until nothing was left but a mass of spheres. These grouped themselves about the young oögonia that had developed from some of the small undifferentiated cells lying along the wall of the ovary

(Plate 3, fig. 1). At this stage the oögonia had a large germinal vesicle, while the cytosome was filled with fine granules and occasionally contained a few small bodies that stained like the larger yolk-forming bodies or nutritive spheres that surrounded it.

GROWTH OF THE OÖCYTE

The follicle cells and the oöcytes seem to have a common origin in the small undifferentiated cells which lie in the wall of the ovary. We have found nothing that explains the divergent development of these cells.

As development proceeded, the nutritive spheres grouped themselves about the oögonia. They were aggregated loosely in a structureless medium, but did not form a true cellular follicle. In some instances the original wall of the cell in which the nutrient spheres were formed persisted. In other instances all traces of cellular structure had disappeared (Plate 3, fig. 2). As the oögonia enlarged there was a corresponding increase in size of their germinal vesicles and nucleoli. The formed components were more numerous in the cytoplasm of these cells than in the younger oögonia, and material seemed to be in the process of passing through the cell wall and into the cytoplasm of the developing oöcyte. As in the earlier stages (Plate 1, fig. 3), all the spheres were granular, but showed wide differences in staining capacity. Many oögonia were completely filled with spheres, although the germinal vesicle was still visible (Plate 3, fig. 3). These growing oöcytes were easily distinguished from cells undergoing disintegration, as their cytoplasm was more homogeneous and their germinal vesicles were still intact. The structures which we have interpreted as nutrient spheres, that have been included or incorporated, lost their identity as such and the bodies disappeared, leaving only granules distributed throughout the cytoplasm. Whether or not the nutrient spheres contributed directly to the contents of the germinal vesicle is not certain. While the deutoplasmic granules were not observed in the actual process of migration into the vesicle, many cells were found with germinal vesicles containing granules that showed the same affinity for stains as similar granules in the cytoplasm. During the process of assimilation, the germinal vesicle often put out irregular projections, and it is probable that this behavior was associated with the formation of yolk.

By the time that assimilation was complete, the oögonium had increased greatly in size; the granules were diffuse, giving the cytoplasm a homogeneous appearance, and were somewhat aggregated around the germinal vesicle, which had reached its maximum size (Plate 5, fig. 1). Preparatory to maturation, a coarse beaded spireme with large chromatic knots appeared in the germinal vesicle, the nucleolus being still visible. In the material available it was impossible to follow the progress of maturation completely. Although chromosomes were seen in many eggs, definite spindles were not found; consequently the formation of polar bodies could not be observed. In one preparation showing a polar body (Plate 5, fig. 2), the nuclear wall had again been formed, although the chromosomes had not yet disappeared, and the nucleus was small, showing that maturation must have taken place.

All oöcytes at this stage showed the deutoplasmic granules distributed throughout the cytoplasm. With the maturation of the egg the granules had gradually decreased in size and were now smaller than in the young oögonia. The decrease in size was made more apparent by the diffuse state of the granules and by the increased size of the egg.

In the mature egg (Plate 6, fig. 2) the granules were evenly distributed and the cytoplasm was homogeneous, although along the wall it was more densely granular. The cell wall, or egg membrane, showed an increased thickness. The nucleus was small and finely reticular. The nucleolus was distinguished by its specific staining reaction.

SUMMARY

1. In *Echinometra lucunter*, undifferentiated cells along the wall of the ovary are uniform in appearance, although they are destined to develop into two entirely different kinds of cells.
2. As development proceeds, some of the cells become oögonia, while others enlarge and disintegrate to form deutoplasmic or yolk-forming bodies. These bodies are more coarsely granular than was the cytoplasm from which they were formed. The granules exhibit different affinities for stains.
3. Fully formed nutritive spheres are of two kinds, granular and non-granular. The former are composed entirely of cytoplasmic, or of both cytoplasmic and nuclear material.
4. The nutritive spheres group themselves around the oögonia in a follicular arrangement.
5. Eventually the nutritive spheres enter the egg and disappear as such, forming the yolk content of the cytoplasm, which becomes homogeneous and evenly granular.
6. The deutoplasmic granules are smaller and more diffuse in the mature eggs than in the oöcytes.
7. The nutritive spheres have been shown to be composed of phospholipins suspended in a homogeneous medium. It is probable that they are largely lecithin in content. Their origin is not known.

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EXPLANATION OF PLATES

All drawings were made with the Zeiss 10x objective and 90 (1/12″) oil immersion lens and the camera lucida adjusted to a magnification of 2340 diameters. Drawings were made only from stages that could be followed through several sections.

ABBREVIATIONS

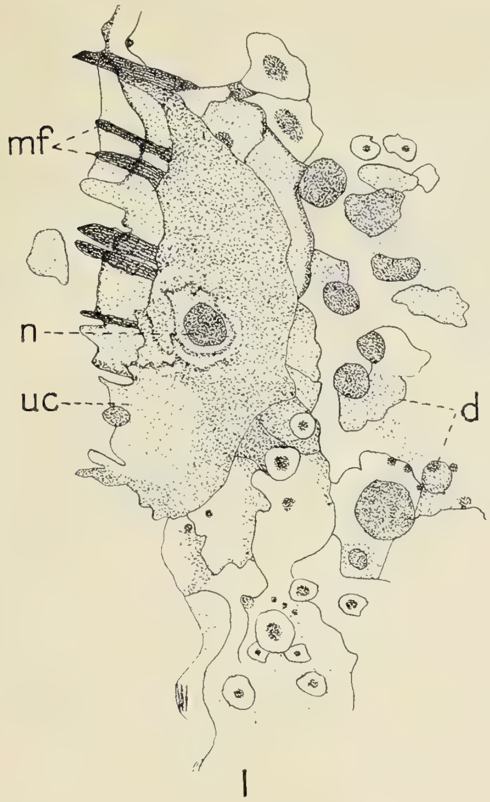
uc.—undifferentiated cell
d.—yolk-forming bodies, nutritive spheres or deuto-plasmic bodies
mf.—muscle fibers
n.—nucleus
c.—cytoplasm
nm.—nuclear material
ow.—ovary wall
gv.—germinal vesicle
no.—nucleolus
ck.—chromatin knots
pb.—polar body
g.—deutoplasmic granules

PLATE 1

FIG. 1. Oblique section of cell in undifferentiated state with nucleus and surrounded by yolk-forming bodies, showing muscle fibers of ovary wall extending in at lower level. (Bouin fixation, stained with iron hematoxylin and eosin.)

FIG. 2. Beginning disintegration of cytoplasm of large cell to form deutoplasmic bodies, no evidence of germinal vesicle. (Bouin with iron hematoxylin.)

FIG. 3. Further stage of disintegration of cytoplasm, no evidence of germinal vesicle. (F. W. A. with Benda.)



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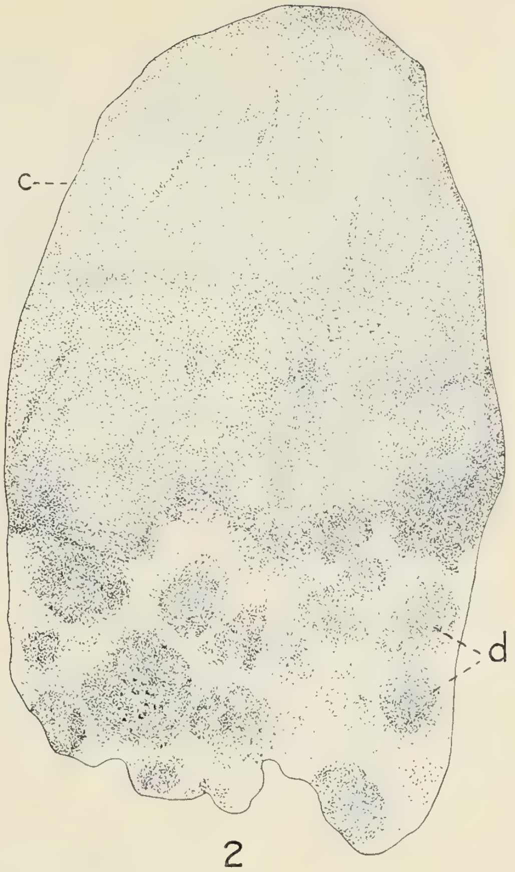

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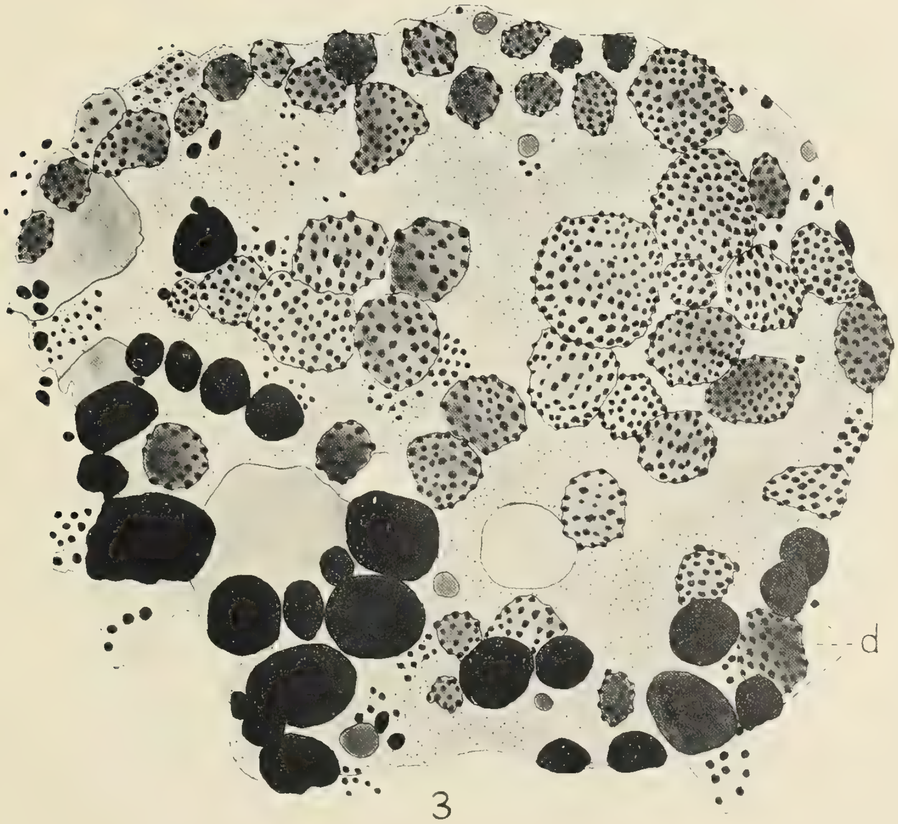

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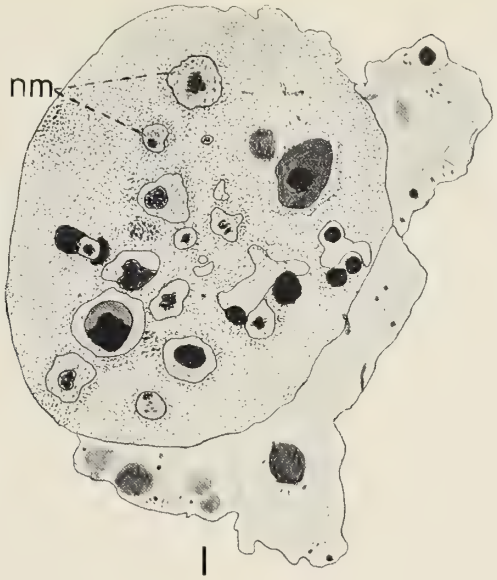
PLATE 2

FIG. 1. Early disintegration of nuclear material of cell to form deutoplasmic bodies, giving appearance of syncytium. (F. W. A. with Benda.)

FIG. 2. Further stage in nuclear disintegration, showing deutoplasmic body formation. (Bouin with iron hematoxylin.)

FIG. 3. Portion of ovary wall showing fully formed deutoplasmic bodies after complete disintegration of large cells. Granular and non-granular bodies may be distinguished, the former containing only cytoplasmic or both cytoplasmic and nuclear material. Remnants of cells are still discernible. (F. W. A. with Benda.)



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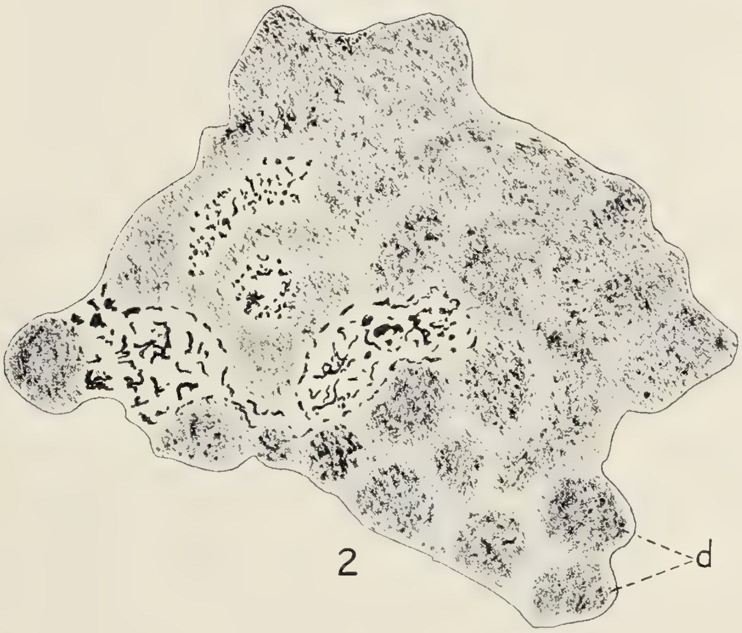

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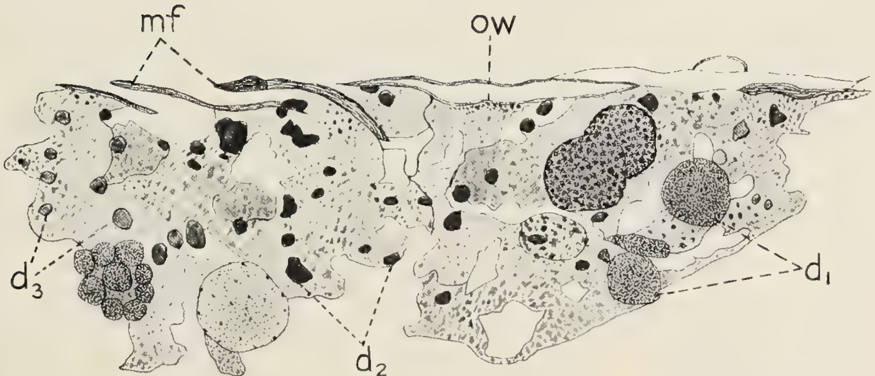

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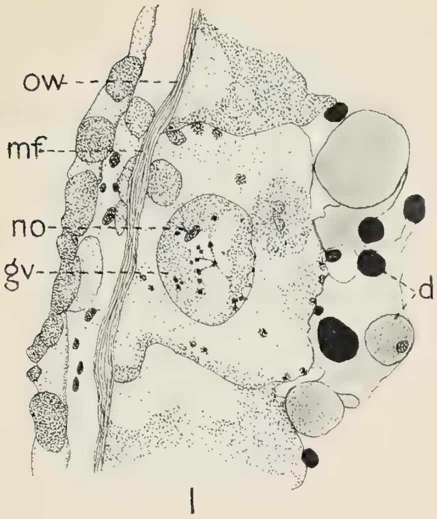
PLATE 3

FIG. 1. Young oögonium along ovary wall with germinal vesicle and nucleolus, showing surrounding yolk-forming bodies. Cells of adjacent ovary wall are shown to left of muscle fibers. (F. W. A. with Benda.)

FIG. 2. Later oögonium with germinal vesicle and nucleolus, surrounded by follicular arrangement of cells, some of which are entering the cytoplasm. (F. W. A. with Benda.)

FIG. 3. Egg completely filled with yolk-forming bodies, germinal vesicle visible. (F. W. A. with Champy-Kull.)



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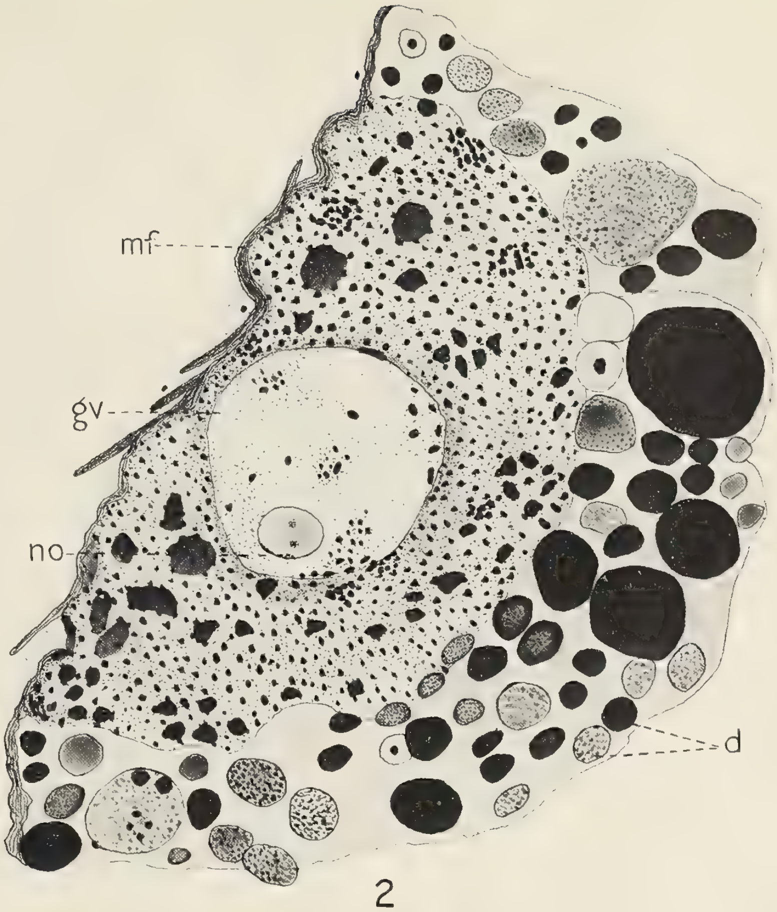

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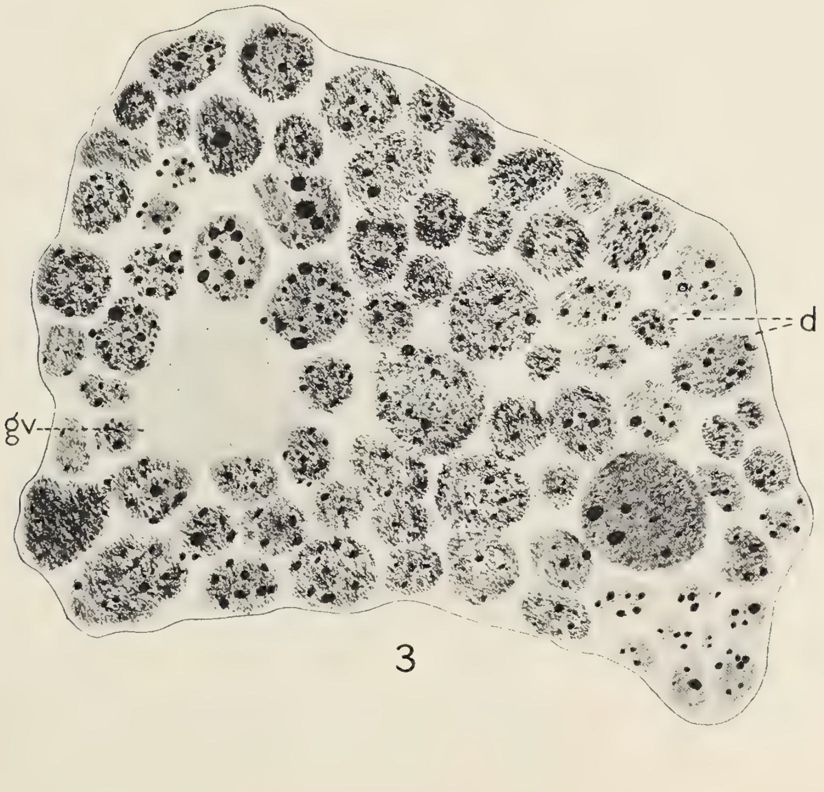

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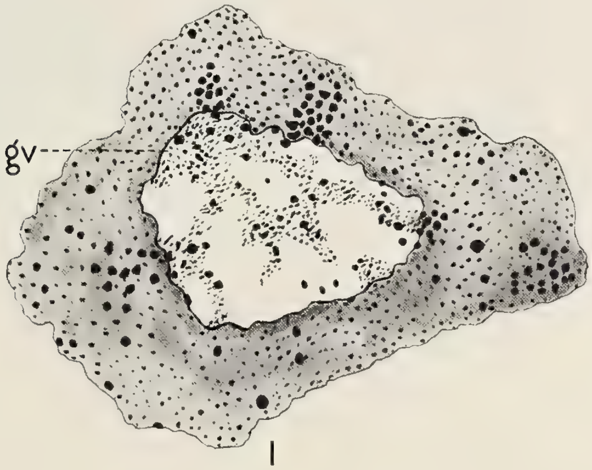
PLATE 4

FIG. 1. Complete assimilation of yolk-forming bodies, showing migration of deutoplasmic granules into germinal vesicle (?). (F. W. A. with Benda.)

FIG. 2. Late oögonium showing irregular germinal vesicle with nucleolus and few remaining yolk-forming bodies. (F. W. A. with Champy-Kull.)



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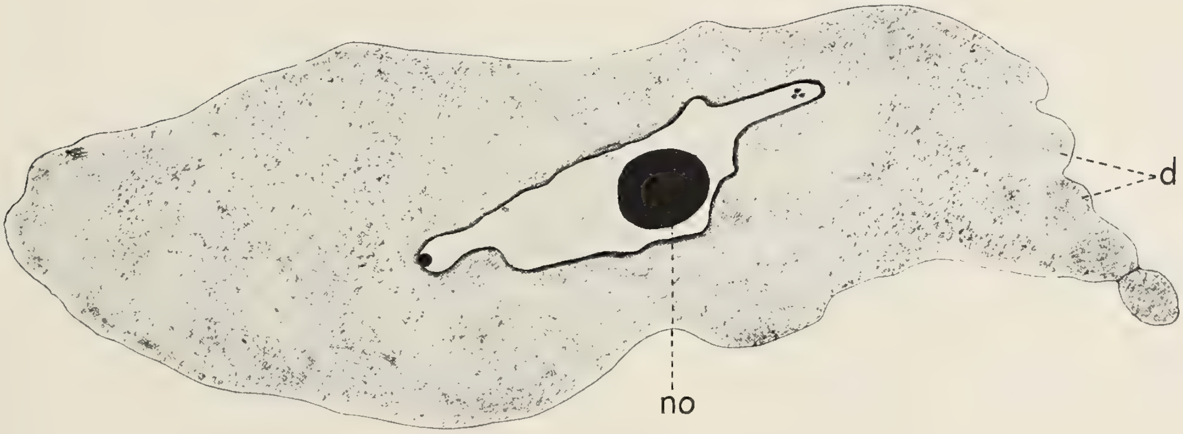

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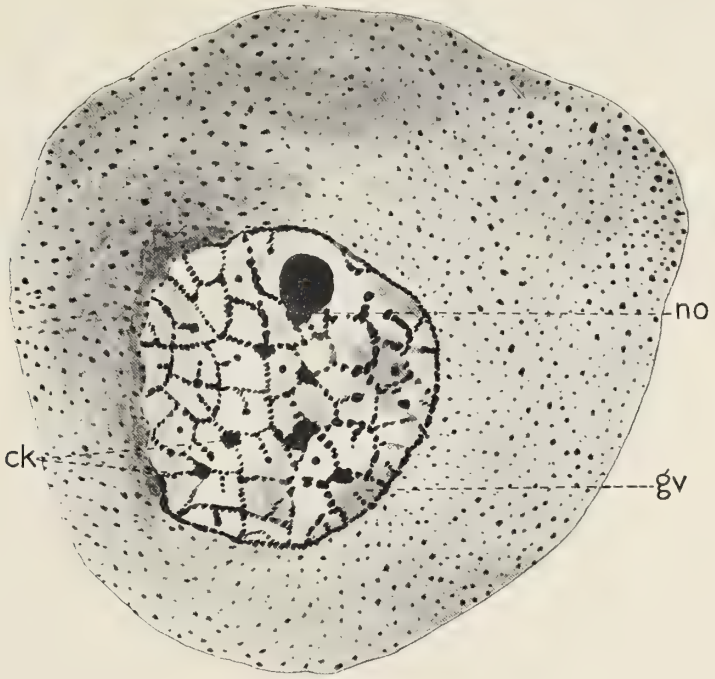
PLATE 5

FIG. 1. Oögonium at beginning of maturation, showing congregation of granules around germinal vesicle and formation of spireme, with chromatin knots and nucleolus. (Bouin with iron hematoxylin.)

FIG. 2. Egg showing polar body and reappearance of nucleus with chromosomes. Nucleus is inserted from another section of same egg. (Bouin with iron hematoxylin and eosin.)



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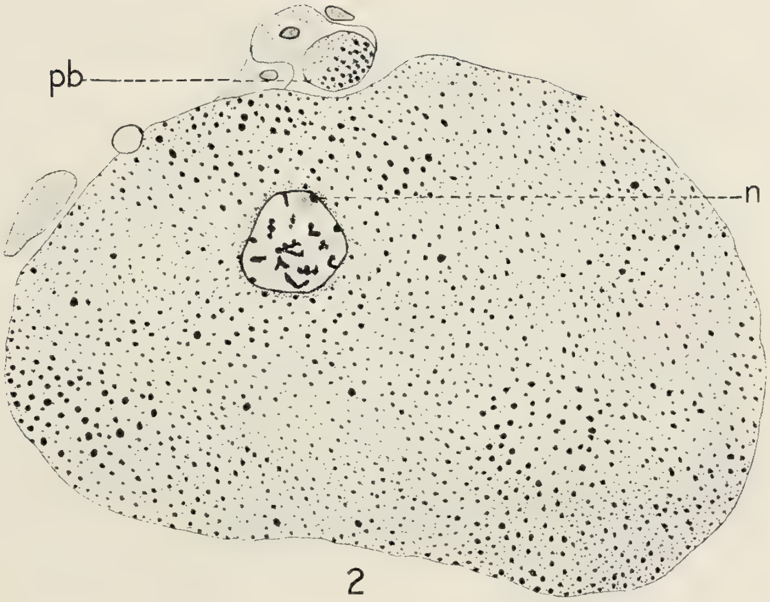

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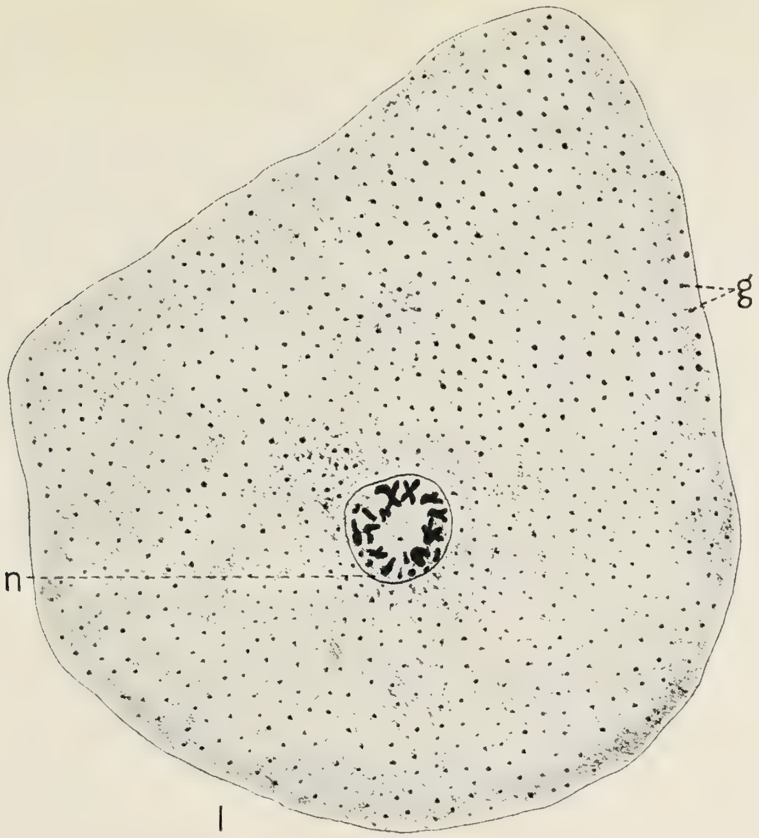
PLATE 6

FIG. 1. Egg with reduced nucleus, showing tetrad-like structures, cytoplasm containing deutoplasmic granules. (Bouin with iron hematoxylin and eosin.)

FIG. 2. Mature egg showing resting nucleus with nucleolus, cytoplasm homogeneous and heavier along cell wall. (F. W. A. with Benda.)



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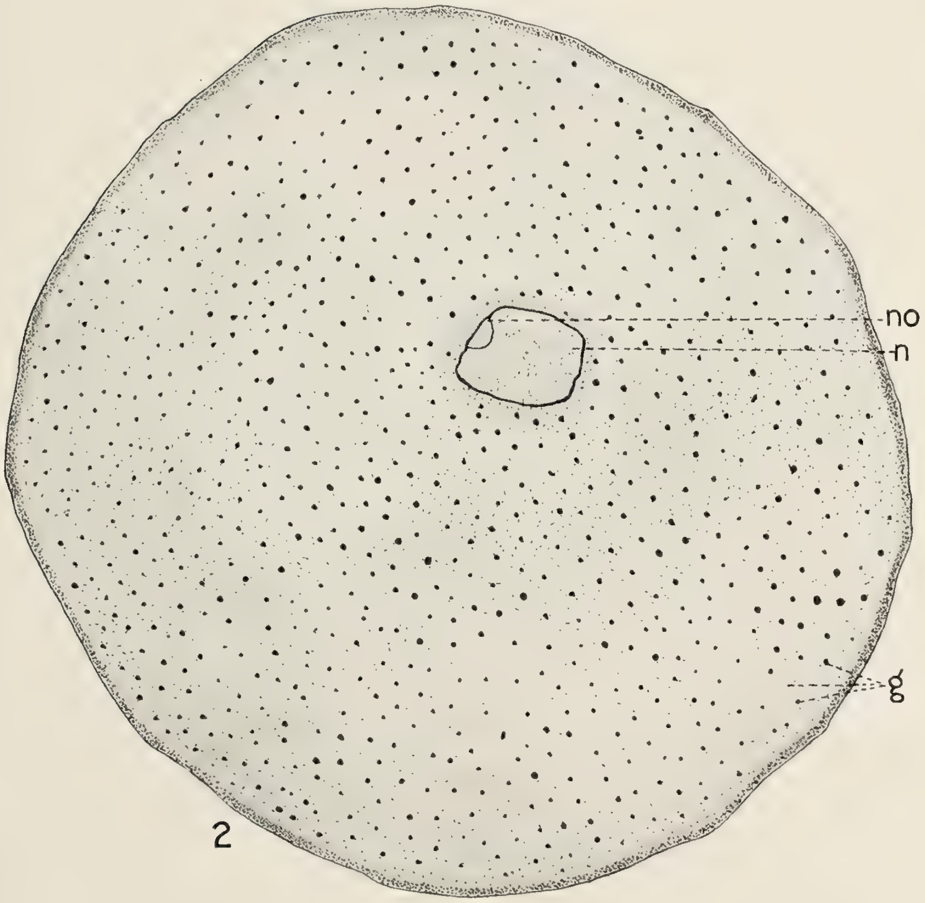

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III

STUDIES ON THE CORAL REEFS OF TUTUILA, AMERICAN SAMOA WITH SPECIAL REFERENCE TO THE ALCYONARIA

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With seven plates and fourteen text-figures

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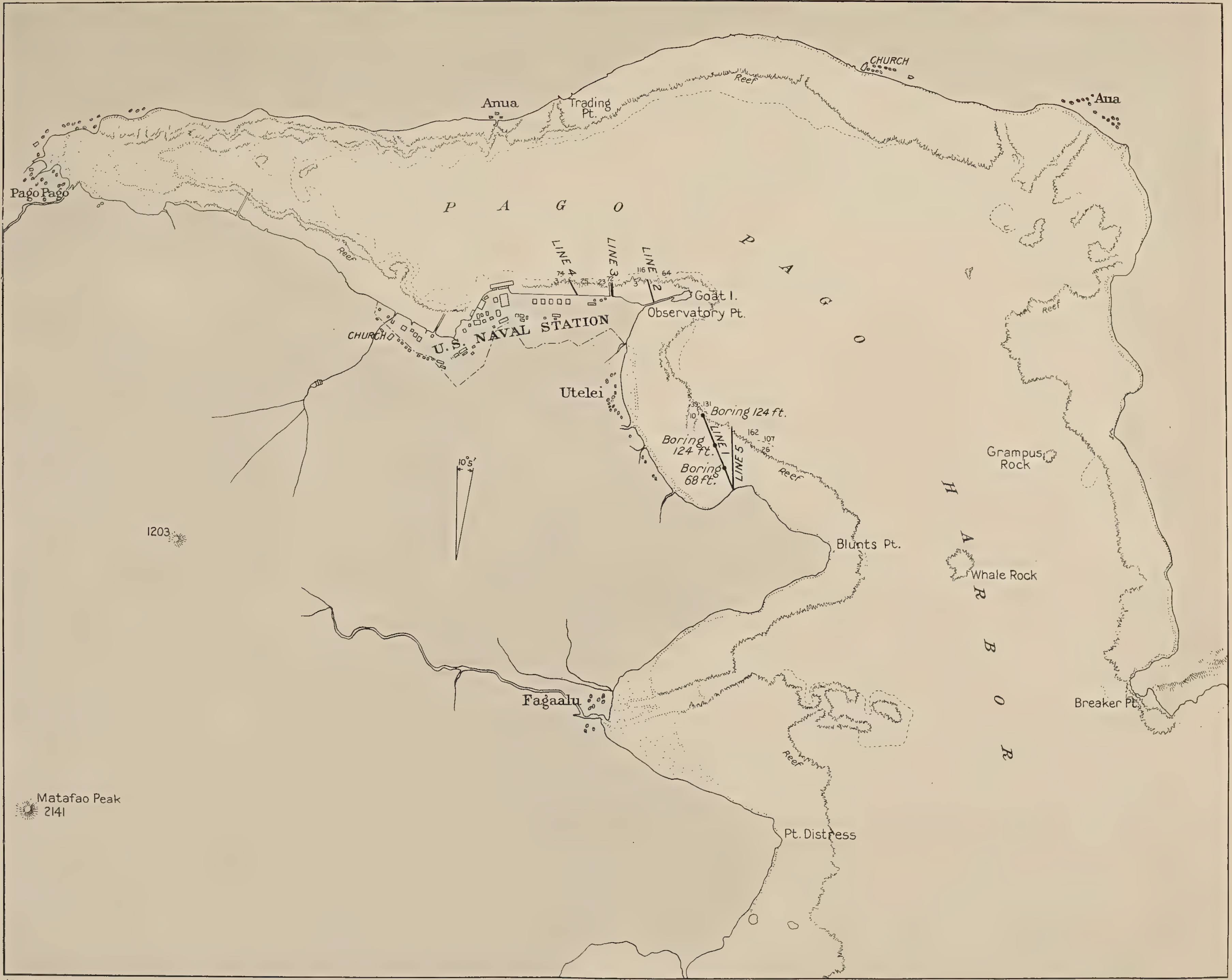
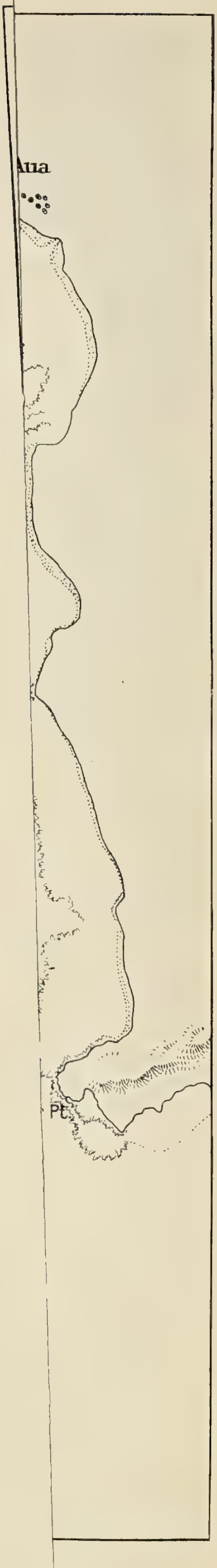


(*From United States Hydrographic Office, Chart 2503*)

MAP OF PAGO PAGO HARBOR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STUDIES ON THE CORAL REEFS OF TUTUILA, AMERICAN SAMOA, WITH ESPECIAL REFERENCE TO THE ALCYONARIA

INTRODUCTION

The following studies on the coral reefs of Tutuila were carried on while the author was a research associate of Carnegie Institution of Washington.

Of the late director of the Department of Marine Biology of this institution, Dr. Alfred G. Mayor, I wish to here express my appreciation of his unfailing sympathetic interest in my work. He provided everything necessary in the way of apparatus and assistance and was especially kind in preventing a break in my records by making observations and measurements of some of my specimens in the summer of 1919, when it was impossible for me to visit the region.

Because of the results obtained from ecological studies on Alcyonaria and their importance in the formation of coral reefs in the Florida-Antillean region, it seemed desirable to extend these observations to Pacific reefs to determine whether or not these organisms are there as important in reef limestone formation as in certain localities on the Florida reefs.

The first season was devoted chiefly to a study of alcyonarian ecology. It then appeared essential to ascertain in so far as possible whether or not these organisms had in the past been as important in reef building as they are now. Since the study of material obtained by boring through the reef with a core drill offers the best opportunity for this determination, three borings were made through one of the reefs where the present conditions had been most intensively studied. As a consequence, this report naturally falls into three divisions: first, the ecology of the Alcyonaria now growing on the reefs; second, the structure of the reefs as shown by study of the surface rock, the cores from drilling, and material blasted at the reef edge; and third, the bearing upon theories of coral reef formation of the relation of reefs in Pago Pago harbor to their substratum. Unforeseen developments have delayed preparation of this report. Publication of papers dealing with phases of the development of the coral reefs of Tutuila (Mayor, Daly, Chamberlin, Setchell 1924) now necessitates changes from its original form.

PART I

ECOLOGY OF THE ALCYONARIA ON THE REEFS OF PAGO PAGO HARBOR

For the practical purpose of this report the general geographical features of the island need to be mentioned in the briefest manner only.

A fringing reef extends out for some distance along the southern shore and along the north shore of the eastern part of the island. In the western part of the north shore, reefs occur in the harbors only. For many practical reasons the exposed outer reefs are difficult to study in detail. Those within the harbor show the entire range of ecological conditions. At the harbor entrance they are fully exposed to the open sea. Toward the inner ends of the bays the influence of fresh water and silt from the streams progressively inhibits and finally prevents their growth.

Since, as a matter of convenience, my extended observations were confined to the reefs within Pago Pago Harbor, more careful consideration must be given to its geographical features.

This harbor occupies what appears to be chiefly a drowned valley, with possibly one or more broken craters near its inner end. From the entrance the channel extends about 1.5 miles in a northerly direction (342°) where it makes a sharp turn to the west and extends for about the same distance, to end at a small delta plain at the entrance of Pago Pago stream. Along the outer portion of the harbor the eastern shore line is relatively straight, and a broad reef extends from Breaker Point to the entrance of the stream at Aua village where there is a well-defined "entrance" through the reef. As this reef is placed with its long axis parallel to the channel the incoming waves run, to a considerable extent, along the reef so that there is a strong current over its surface parallel to the shore line.

On the west side of the outer portion of the harbor the shore line is much more broken. At Fagaalu, about one-third of a mile inside the harbor entrance, there is a deep cove into which flows one of the larger streams of the island, so that the reefs in this cove are separated into a northern and a southern portion, with a rather wide, barren stretch of sandy shore between. The southernmost of these reefs is continuous with the fringing reef on the south shore of the island, and has full exposure to the unbroken ocean waves. At its inner end the reef on the north side is well protected so that severe wave action occurs only in times of heavy storms.

On the north side, Fagaalu Cove is limited by Blunts Point, a sharp promontory that protects from direct wave action the deep cove lying next inward from the entrance. As is true of all the coves about the harbor, this one, at the inner end of which is situated the native village of Utelei, represents a branch valley opening into the larger valley which now constitutes the harbor. At its inner end there enters a small stream that brings enough fresh water and sediment to prevent the growth of corals and thus provides an entrance through the reef (figs. 1 and 2).

The reefs on the two sides of the cove at Utelei village were the site of the greater part of my more extended studies and will in future be referred to as the Utelei reefs. As the borings and the most important lines across the reef, where area covered by growing Alcyonaria or Alcyonarian spicule rock was determined, were situated on the south side of the cove, all unqualified references to the Utelei reefs will designate this particular locality.

On this reef, which is continuous around Blunts Point with the north reef of Fagaalu Cove, there are to be found all gradations of environmental conditions from practically full exposure to the most severe wave action occurring within the harbor, to quiet water at the western end of the cove at the sand flat where the stream enters.

Between the north and south reefs is a channel the bottom of which is, in its inner portions, entirely covered with growing coral so that it is possible in this protected locality to study the vertical as well as the horizontal distribution of the reef-forming organisms. The distance across this reef in a straight line from the shore to its outer margin is 945 feet at the widest point. This line extends from the north side of Blunts Point to the channel between the two Utelei reefs and marks the location of one of the series of squares where the relative area of reef surface covered by Alcyonaria was determined, and where the borings through the reef were made (line No. 1, fig. 1). From the same point of origin a line extending to the margin on the east side of the reef was 625 feet in length (line No. 5, fig. 1).

The reef on the north side of Utelei Cove is continuous with the reef extending along the south side of the inner portion of the harbor beyond the abrupt bend to the west. Goat Island, an off-lying fragment of basalt, rises some 40 feet above the surface of this reef at the turning point of the channel and is separated from the main island by a distance of about 100 yards.

The reef along the south side of the inner harbor has been so greatly influenced by changes made in connection with the development of the United States Naval Station that its original character has been destroyed over the greater part of its extent. For a considerable distance inward from the point opposite Goat Island a wide fill has been made, which for some distance extends outward to the original reef border. This fill and the change in drainage attendant thereon has so altered the natural conditions that, over wide areas, nothing remains but barren reef where all growing corals have been destroyed as a result of some disturbance in the environment to which they had become adjusted.

Farther in the harbor, near where the natural limits of the growth of reef building organisms is reached, the conditions appear to be less affected as a result of human activities and scattered specimens of the more resistant organisms are still to be found.

On the north side of the inner harbor reef patches first appear about 0.75 mile from the entrance of Pago Pago stream. The growth of reef-forming organisms becomes progressively more abundant outward, until a continuous reef is established. This reef extends along the north side of the harbor, broken here and there by very narrow tortuous entrances at points where small streams come down from the hills. Starting at its western end as a series of scattered coral patches, this reef reaches a width of approximately 1000 feet near its eastern end where it is fully exposed to the clear, well-aerated and food-laden water coming straight in from the harbor entrance some two miles away.

With minor changes this description of the conditions within Pago Pago outer harbor would apply equally well to those existing in the many bays or harbors about the island. In some instances, the bays are broadly open to the ocean and have no protected coves. Where no stream with a constant flow of water occurs at the head of a bay, there are no breaks in the reef, but these conditions are of very rare occurrence. At Nuuli a lagoon lies behind a barrier reef, but this condition has been brought about by the occurrence of a lava flow that took place after the modern reefs had been formed. It extended to the outer edge of the reef, leaving only a narrow entrance to the bay behind the Nuuli sand spit, which itself has apparently been formed, in part at least, through the action of currents set up on account of the changes brought about as a result of this laval flow. Since the original entrance to this bay has been blocked by the laval flow and no well-defined channel has been formed to take its place, the conditions found here constitute a special case which is not duplicated elsewhere about the island. Because there is no rapid interchange of water between the inner lagoon and the ocean except at highest tide, the whole area is wanting in organisms which require clear, well-aerated water and has a silt-covered bottom; while about the shores, which are rapidly encroaching on the area of the lagoon, there occurs by far the largest of the few mangrove swamps found about Tutuila.

Since the ecological conditions to be found in Pago Pago harbor are so typical of those occurring about the island generally, and since it was most practicable to carry on detailed studies near our base of supplies, the work done elsewhere was essentially to afford comparison with the results obtained where the factors had been studied more completely. In general the observations made elsewhere than in Pago Pago harbor will not be discussed under their locality designation, but it can be safely assumed that the statements made concerning this circumscribed portion of the reefs are applicable to a corresponding reef area anywhere about the island.

CHARACTER OF THE PHYSICAL ENVIRONMENT OF THE UTELEI REEFS

As limited for the convenience of this study, the Utelei reefs may be taken as starting from the apex of Blunts Point, and extending around the cove to Goat Island at the point where the harbor channel makes the nearly right-angled bend to the westward. Within the area thus delimited practically the entire range of physical conditions to be met anywhere about the island occurs, with one marked exception—that nowhere was there found many coral skeletons cast up beyond high-tide mark by wave action during hurricanes, although such are to be seen at many points along the exposed outer coast of the island and in some of the shallow bays on the north shore. On the other hand the violence of wave action to which the most exposed portions of this reef are sometimes subjected is attested by the presence of several so-called "negro-heads,"—large fragments of the reef margin which have been upheaved by the force of the waves and carried inward for some distance on to the surface of the reef.

At the other extreme as regards wave action, stand the conditions found at the inner end of this reef near the bight of the cove. Not even during the most severe storms in the three seasons over which my studies extended, waves were observed of sufficient violence even to stir up thoroughly the loose sand with which this portion of the reef is covered except where growing organisms are attached.

Because of the character of the shore line, the currents over the outer portion of this reef run nearly parallel with its margin (*i. e.* northwest) and have the greatest velocity. Some distance north of Blunts Point the currents, as their velocity decreases, are deflected toward the shore, so that by the time they have reached the deeper portion of the cove they run parallel to the reef border at nearly a right angle to their original direction. At the same time as the force of the waves is spent as they run inward from the harbor entrance along the border of the reef, their direction is altered by contact with the reef, so that to an increasing extent they are turned inward across the reef. At a point where the border of the south reef faces west of north, as at the outer end of line 1 (see map), the direction of the waves at the reef margin has been turned through 90° from their original direction. As a consequence of these conflicting directions of wave action, "tide rips" occur at many points on the surface of the reef whenever the tide is above half flood.

Besides these changes, the direction of the currents is constantly altering with the rise and fall of the tides, so that never except at identical tidal phases will the currents be of the same direction and intensity at any given point. When the tide is near full flood—and especially when the water is "backed up" in the harbor as the result of unusually heavy winds from the southward—each wave traverses nearly the entire width of the reef without losing its identity. At even neap low tides some portions of the reefs are exposed and the waves break at the reef margin so that only a small volume of broken water makes its way along the tortuous lines where the surface of the reef is lowest.

During the few days of spring tides a large proportion of the reef surface is exposed for a period of two hours or more, and at this time on quiet days the reef becomes empounded, since the Lithothamnium ridge at the reef margin is always several inches higher than the general reef surface. Near the inner edge of the reef, where few living corals occur, the sand has been scoured out by the currents to such an extent that even when the reef is empounded the depth of water will here be at least 12 inches.

The velocity of the currents over different portions of the reef surface was determined at several locations and under different conditions of tides and weather. The observations were made by determining the distance a floating object, as the midrib of a cocoanut leaf, would be carried in one minute. Near the inner edge of the reef, at low tide the velocity was on the average 15 feet per minute. Toward the reef border, wherever the water was of sufficient depth to permit such determinations, the currents varied in velocity from 25 to 50 feet per minute. As the tide rises, the velocity of the currents constantly increases, until the water has reached such a depth that when a wave reaches the border of the reef it continues as an overhanging breaker which rushes across the reef at a speed frequently as great at 175 feet per minute.

TABLE 1—*Line 1, July 22, 1920. Very low tide but heavy swell*

Distance from shore	Depth	Temperature	Current	Remarks
feet	*inches*	°C	*knots*	
25	4	30.3	0.48	Many places were shallower than on my line
100	14	28	.40	
200	10	29.2	.50	
300	10 to 20	29.2	.60	
400	12	28.1	.25	
500	10	28.2		
600	16	28.05	Irregular, dependent on breakers.	
700	9	28		
800	12	27.9	Very strong at each breaker, then back set between waves.	
900	..	27.95	Very strong, each breaker at rate of 1.5 knots, then slack between waves.	Lithothamnium covered intermittently.

When the depth of water over the reef has become such that ordinary waves no longer break at the reef margin, the currents across the reef decrease in velocity until at full tide their rate will have fallen to not more than 30 feet per minute over the entire reef. At this time the direction of the currents is much more constant and less influenced by the shore lines and irregularities of the reef surface. In general, during the period of high tide, their direction conforms to the long axis of the outer harbor rather than to the outlines of the shore or reef margin.

With a rise and fall of the ordinary tides of roughly 3 feet, the temperature of the water over the reef necessarily shows great variation from time to time. When the low-tide period falls within the hours of intense sunshine, the variation in temperature naturally shows an extreme range. With the temperature of the water in the channel off these reefs ranging only slightly from 29° C., an increase of as much as 7° C. was sometimes found when the reefs were empounded near midday. This temperature, if continued for as long as one hour, is fatal to most of the organisms found on the reefs, so that the death of many corals from asphyxiation is a not uncommon occurrence under the extreme conditions prevailing when the low spring tides fall at midday. Under the less extreme conditions usually prevailing, the range of temperature caused by differences in the depth of water over the reefs and differences in the velocity of currents is not more than 3° C. from that of the water in the channel and is well within the range favorable for the metabolism of the Alcyonaria as well as other reef-dwelling animals.

The conditions at average low tide are shown in table 1 and appended statement.

When the tide had risen about 1 foot from its unusually low condition, the water making up the breakers traveled across the reef in a generally south westerly direction at a rate at times as high as 7.5 knots per hour over the outer third of the reef.

CHARACTER OF REEF SURFACE IN RELATION TO OCCURRENCE OF ALCYONARIA

In general the character of the reef surface is such that it affords a favorable place for the attachment of Alcyonarian colonies. On most of the reefs, however, except some of those about the exposed portions of the island, there was near shore an area of varying width composed almost entirely of shifting sand where, of reef-forming organisms, the massive *Porites* and *Sclerophytum confertum* alone seemed to find suitable environment. Between this area and the Lithothamnium ridge the entire reef surface, whether or not exposed at low tide, would appear to afford a favorable place of attachment for reef-forming organisms, and their absence from some locations must be attributed to factors other than the nature of the reef surface.

As practically every student of coral reefs has noticed, the proportion of alternately exposed and submerged or constantly submerged reef surface occupied by living reef-forming organisms is surprisingly small, and the most careful study of the environmental conditions fails to throw any light on the reason why one area is densely carpeted with living organisms while an immediately adjacent spot, where every factor that can be determined is identical, remains barren over extended periods.

Beyond the rim of the shallow portion of the reefs the proportion of barren area is very much smaller.

The bottom of the channel between the two reefs at Utelei, in depths less than 25 feet, to which depth it was carefully examined by the use of a diving hood, was so covered with living coral-forming organisms that barren spots were rare and of very limited extent.

On the surface of the reef, where at definite phases of the tide heavy breakers run across, it would be essential for their early development that the planulæ of Alcyonaria or stony corals should find lodgment in crevices where they would for a time be protected from the violent agitation of the water and attrition from shifting sand. While this necessity might restrict to some extent the distribution of these organisms over the reef surface, it does not account for the presence of adjacent barren and fully covered areas.

SPECIES OF ALCYONARIA OCCURRING ON REEFS OF TUTUILA

Dana (1846), in the report of the Wilkes Expedition, lists five species of Alcyonaria from Tutuila. He refers all of these to the genus *Alcyonium*, although his figures are sufficiently accurate to enable one readily to establish the identity of the forms to which he had reference (see Pratt ('06), Kukenthal ('03) *et al.*).

Collections made at many points about Tutuila, from the surface of shallow reefs, from the submerged reefs in relatively shallow water, and by means of a dredge from all parts of the harbor, added to this list only a single species—a fleshy Nephthyid from deep water.

Indeed one of the forms recorded by Dana is found so rarely, that for all practical purposes the alcyonarian fauna may be considered composed of four species only. Of the four common species, *Sclerophytum confertum* Dana and *Sclerophytum densum* Whitelegge are by far the most abundant. Both form a skeletal mass of cemented spicules at the base of the colony which bears the fleshy portions, much as in the stony corals the living tissue is supported by the calices.

In *Sclerophytum confertum* the living tissue commonly separates into units of varying sizes, each of which forms at its base a mass of spicule rock. When this species is growing where there is an accumulation of sand, the upward growth of the colony, keeping pace with the elevation of the reef surface, causes the formation of a branching, tree-like mass of spicules which may extend for several feet below the reef surface. Colonies of this type are illustrated in figures 16 and 17, plate 6.

On a hard substratum nearly all the larger colonies have a central barren area composed of spicule rock. As the colony increases in size, this barren area comes to occupy a proportionately larger space. Since spicule formation in the central area occurred for a shorter time than about the borders of the colony, there is always a depression in the center and either a living colony or the skeleton from which all living tissue has been removed is bowl-shaped.

In striking contrast to this ordinary type of colony there sometimes occur masses of spicule rock formed by *S. confertum*, which rise from the reef surface at considerable depths and extend to low-tide level. Most of such masses consist of a branching and anastomosing skeletal framework with the apex of each branch crowned by living tissue. Some of the colonies of this type were 30 feet in circumference and 5 feet high.

When a colony of this species has lived for a relatively short time only, it leaves after its death a skeleton of the character illustrated by figures 17 and 18, plate 7. Without reference to their texture, which on their being broken shows plainly the large spicules of which they are composed, these structures are so characteristic in appearance that they constitute one of the striking features of the limestone on the reef surface wherever this species occurs abundantly. Very frequently such skeletons are found broken loose from the substratum and being rolled about over the reef surface by currents just as are *Fungia* skeletons, which in a rough way they resemble.

The colony form of *Sclerophytum densum* may be best described as characteristically procumbent. Although under normal conditions it fits itself to the irregularities of the substratum upon which it occurs, upright, branching colonies, so characteristic of *S. confertum*, are never formed. Beneath the older colonies the limestone composed of their cemented spicules may reach a thickness of 18 to 24 inches when they occur in the most favorable locations. Under such circumstances, however, the colony is raised as a whole above the general reef surface and the skeleton constitutes a solid mass.

Rarely, under the influence of some special stimulus, a colony takes on a cylindrical form and grows upward from the reef to a considerable height. In every instance where such colonies were carefully examined the skeletal axis was found to be penetrated by the tubes of an annelid. These serpulids would under normal conditions be found with their cirri extended from the apex of the alcyonarian colony, and they gave the casual observer the impression that they might well be the cause of the unusual growth form of the organism in whose tissues they had taken up their abode. The cylindrical colonies were observed in deeper water only, and several examples more than 10 feet high were measured.

Since there is ordinarily no separation of the colony into smaller units, and the death of a definite part of the colony does not regularly take place, there is formed by continued growth a solid sheet of tissue which persists for a considerable period.

TABLE 2—*Percentage of spicules to fresh weight*

Specimen	Fresh weight	Weight in spicules	Percentage of spicules to fresh weight
Sclerophytum confertum:	*grams*	*grams*	*p. ct.*
1	1162.35	311.85	26.829
2	1559.25	269.32	17.24
3	595.35	155.92	26.19
4	1927.80	496.12	25.73
5	992.25	141.75	15.71
6	113.40	28.35	25.00
			22.783 (average)
Sclerophytum densum:			
1	311.85	127.57	40.90
2	467.77	155.92	33.30
3	240.97	70.87	34.00
4	155.92	56.70	36.40
5	123.57	42.52	33.33
			35.58 (average)
Sarcophytum latum:			
1	1176.52	70.87	6.20
2	496.12	42.52	8.56
3	453.60	42.52	9.40
4	567.00	42.53	7.51
5	56.70	7.08	12.00
			8.73 (average)
Nephthya flexile:			
1	1048.95	127.57	12.15
2	708.75	70.87	10.00
3	652.05	42.52	6.50
4	737.10	70.87	9.65
5	311.85	28.35	9.10
			9.48 (average)

In none of the specimens used for growth records extending over 3 years was there any evidence of the breaking up of the colony or of the death of any particular portion. Because of retention of continuity, the colony and its constant increase in extent, large areas come to be overgrown by this species to the exclusion of all other large organisms except those that find a suitable place for attachment in the grooves between adjacent alcyonarian colonies and extend beyond the reef surface, as do the branching *Porites* and *Acroporas* among the stony corals.

While the period of three seasons covered by my observations was apparently not of sufficient length to cover the usual span of life of this species, abundant evidence was found on the reefs that within a relatively short time there had occurred over extensive areas the death from some cause, natural or catastrophic, of all *S. densum* colonies. The area of nearly vertical reef surface faced by limestone composed of spicules of this species was in one instance about 0.25 mile in length, and over the entire area living "coral" was found in only a few places, so that the death of the *Sclerophytum* must have occurred within a short time. There is, unfortunately, no criterion by which it is possible to determine the time necessary for the re-establishment of the reef fauna on such a smooth, barren, vertical surface, so that the time since the death of the Alcyonaria may be greater by far than I have assumed from the character of the exposed spicule rock.

The two common species of Alcyonaria remaining do not form solid limestone by cementing together their spicules. One of them, *Sarcophytum latum* Dana, has a soft fleshy body with conspicuous feeding polyps and only very small spicules as skeletal elements. Indeed the spicules are so small and few that they do not make a striking feature of the cut or torn tissues as they do in *Sclerophytum.*

Possessing only spicules of such a character, *Sarcophytum* can not add massive material to the reef limestone, and its contribution will be lost sight of among the particles of sand from other sources with which it will be mixed. In fact it is not by limestone secreted but by its ecological relation to other species which are the important reef builders that this organism must be judged.

Nephthya flexile Dana, a finely branched, fleshy alcyonarian, is the remaining common species. Of the four it has by far the most limited distribution, being restricted to the most protected locations. Like *Sarcophytum* its spicules are small as compared with those of the *Sclerophytum,* and are present in relatively small bulk. Except for the fact that this form occurs in abundance in a few favorable habitats where it is the dominant element of the fauna to the exclusion of more actively lime-secreting species, it might well be left out of consideration. On some other Pacific reefs, notably the barrier reefs about Suva Harbor, Fiji, it covers areas several square miles in extent, and in such cases must be a really important factor in limestone formation.

In table 2 are given the proportion by weight of spicules in the tissues of the four species of Alcyonaria considered in this section.

DISTRIBUTION OF THE DIFFERENT SPECIES OF ALCYONARIA IN RELATION TO ENVIRONMENTAL FACTORS

While all four important species of Alcyonaria appear to find their most favorable environment only on relatively protected reefs (or in deeper water) where they are not subjected to direct action of breakers constantly being formed on the most exposed reefs along the borders of the island, there is still sufficient range in resistance to adverse conditions shown by the several species to cause considerable differences in their distribution upon reefs where all of them occur.

Sclerophytum densum is the least resistant of the four species, *i. e.,* it requires better aerated water. It is consequently restricted to locations where there is least possibility of its being asphyxiated by an increase in temperature of the water or by the deposition of sand or silt upon it.

Such conditions are found on reefs across which there is a strong current when the tide is down and the depth over the reef is slight, and also over the vertical edges of the reefs where sedimentation is impossible and the water is always of approximately uniform temperature. The effect of the first of these conditions is well illustrated on the reef on the south side of the cove at Utelei. Here the heavy swell which always sets into the mouth of the harbor causes a strong current around Blunts Point. This current sets across the Utelei south reef in such a direction that the outer third of line 5 is within its path, while all of line 1, which has the same point of origin on shore, is beyond its effect. During the trade-wind season there is at all times a fairly strong current setting in from the edge of the reef over the area traversed by line 1, but in the hottest part of the year, when the tides are low and the swell in the harbor least, this reef area is sometimes subjected to increase in temperature and also to silting from sand beyond the limits within which *S. densum* can survive. This species is a constant element in the reef-edge fauna of all the reef examined, either in the harbors or exposed.

Sarcophytum latum is nearest to *S. densum* in its incapacity to resist adverse environmental conditions. While not so definitely restricted to areas where the water is deep or the currents strong as is the former species, *S. latum* is usually restricted to the deeper waters about the outer edge of the reefs in the more protected locations, where, on account of the absence of breakers, no Lithothamnium ridge occurs and where no strong currents are developed. On the Utelei reefs, the general distribution of this species follows quite closely that of *S. densum*. Very few specimens are found along line 1, but on line 5 *S. latum* reaches its maximum before *S. densum* becomes represented by more than a very few scattered specimens. (See text-fig. 11.)

Nephthya flexile, which from its distribution appears particularly well fitted to withstand silting, is never found in any location except where it is afforded the utmost protection from violent agitation. It is absent from the surface of all reefs in the harbor except those inside of Goat Island on the south shore. In the cove at Utelei it is abundant in the deeper water over the edge of the reef on the south side, but on the reef along the north side of the cove, which is more directly exposed to wave action, not a single example of this species was found.

S. confertum is by far the most widely distributed species on the Pago Pago reefs. It is the most resistant to high temperatures and to silting, while, on the other hand, its firm texture and the fact that it is attached by a dense spicular mass to its original support allows it to withstand severe wave action without any noticeable detrimental effects. On the surface of every reef this species is found nearest to the shore in shallow water, where the bottom is frequently covered by shifting sand or sediment, where of the stony corals only the massive *Porites* seems able to exist. At the other extreme of reef conditions it constantly occurs along with *S. densum* on the vertical reef borders, although in such locations it seems unable to compete successfully with the latter species, probably because of the more rapid lateral extension of *S. densum* which is a flat encrusting form.

TEMPERATURE EXPERIMENTS

The relative resistance to increased temperature (asphyxiation) of the four important species is shown in the following experiments. The specimens were collected without injury, brought to the laboratory in glass jars of fresh sea-water and immediately transferred to the thermostat. The temperature of the air in the chamber was taken at the beginning of each experiment and that of the water when the period of heating was over. The specimens were then transferred to a jar of sea-water of normal temperature, which was at once taken out and suspended at a depth of about 12 feet from a mooring buoy in the middle of the harbor. The motion of the buoy raised and lowered the jar constantly so that a thorough changing of the water was going on all the time. After from 12 to 36 hours the specimens were examined to determine which of them had survived. This was done by holding them over the side of a moving launch, when the soft tissues would be quickly washed away if the colony had been killed.

All of the forms except *S. rigidum* were, when uninjured by the heating, capable of contracting strongly when treated in this manner, so that the relative extent of the injury caused by different lengths of exposure could be compared on the basis of this reaction.

Experiment 1, April 10, 1917:

Specimens from reef north of Goat Island Bridge.
In thermostat at 35.3° C. for 45 minutes.
Temperature of water at end of experiment, 33.3° C.
S. densum —killed.
S. latum —very badly injured, killed?
N. flexile —severely injured.
S. confertum—apparently uninjured.

Experiment 2, April 12, 1917:

Specimens from reef north of Goat Island.
In thermostat at 35° C. for 30 minutes.
Temperature of water at end of experiment, 33.9° C.
S. densum —killed.
S. latum —somewhat injured.
N. flexile —uninjured.
S. confertum—uninjured

Experiment 3, April 12, 1917 (evening):

Specimens taken from along line 5 and north of Goat Island (*N. flex*).
In thermostat at 35.5° C. for 30 minutes.
Temperature of water at end of experiment, 34.6° C.
S. densum —mostly killed.
S. latum —barely capable of contraction.
N. flexile —slightly injured.
S. confertum—uninjured.

Experiment 4, April 14, 1917 (evening):

In thermostat at 36° C. for 42 minutes.
Temperature of water at end of experiment, 34.9° C.
S. latum —killed.
S. confertum—somewhat injured.

Experiment 5, April 14, 1917:

Specimens from Fagaalu south reef.
In thermostat at 37° C. for 45 minutes.
Temperature of water at end of experiment, 35.7° C.
S. confertum, 3 specimens, all killed, thoroughly expanded.

The behavior of all these species in response to the conditions in the heat experiments corresponds to their reaction to natural environmental conditions as shown by their distribution on the reefs, which is clearly determined by their ability to adjust themselves to unfavorable conditions.

RESPIRATION EXPERIMENTS

The general metabolic activity of the several species considered in the foregoing experiments was determined on the basis of their oxygen consumption, as a check on their resistance to unfavorable conditions, such as asphyxiation due to high temperature or the presence of excessive amounts of sediment in the water.

With organisms of this character, in which a varying portion of the weight of specimens of the different species consists of inert material—the spicules—it has seemed necessary in all cases to reduce the mass used in the computations to the total weight, less the percentage by weight of spicules found to be characteristic of that form. The figure noted in Table 3 as "O_2 consumption per kilogram of living tissue per hour" is of necessity inaccurate because of the uncertainty of the amount of water held in the canals of any alcyonarian colony at any given time. Since the treatment given each specimen from the time it was removed from the reef was identical with that given each of the others, all differences in the results due to this factor would depend upon the physiological state of the specimens and could not be controlled by any practical means.

At the close of each experiment the specimen studied was dried in an oven at 100° C. for 12 hours and the O_2 consumption based on dry weight, corrected for spicule content, compared with that obtained in the original experiment.

With minor fluctuations the two series of results conformed so closely that the first mentioned only is recorded in the following table.

TABLE 3

Species	Number of determinations	c.c. O_2 per kg. of living tissue per hour
Sarcophytum latum	10	19.383
Sclerophytum confertum	23	42.75
Sclerophytum densum	6	23.175
Nephthya flexile	7	74.80

As was determined for several species of Alcyonaria from the Florida reefs (Cary 1918), there is among the Samoan species no definite correlations between metabolic activity and asphyxiation. On the other hand the relation between respiration rate and the proportion of surface to weight $\left(\frac{\text{cm}_2}{\text{gms}}\right)$ observed for the Gorgonaceæ of the Atlantic was in general confirmed by the results recorded above. The very marked difference in respiratory activity between the finely branched *Nephthya* and the practically smooth-surfaced *Sarcophytum* especially emphasizes this relationship. The distribution on the reef (see text-figure 11) of the several species is essentially in inverse relation to the respiration rate. *Sclerophytum rigidum*, the constant inhabitant of especially well-aerated water, has a low oxygen consumption; *S. confertum*, the most adaptable species, stands in an intermediate position as regards respiration, while *Nephthya flexile* which occurs only in the most protected locations, but can best withstand silting, has by far the most rapid respiration.

RELATIVE AREAS OF REEF SURFACE COVERED BY ALCYONARIA

(See map and figure 1, of plate 1 for location of lines.)

Since the distribution of the Alcyonaria on the reefs where intensive studies were made is certain to undergo changes with the passage of time, and especially since the areas studied are easily available for comparison at any future date, their locations have been carefully recorded and the photographs shown in figures 1, 2 and 3 are included to show the bearings of some prominent features of the surrounding terrain, so that the exact location of each line may be accurately determined by anyone interested.

The following lines across reefs in Pago Pago Harbor were laid out and the relative area occupied by Alcyonaria determined by marking off a series of 25-foot squares along the line and making determination of the area covered by Alcyonaria on each of a series of small squares (25 square feet in area) into which each of the large squares was divided.

Line 1: (*Utelei Line*)*:* Extends from survey station "0" on south side of Utelei Cove, N. 32° W., 945 feet to edge of reef.

The following measurements and bearings of conspicuous objects mark point of origin: Compass set up left of survey mark 31 feet west from iron telegraph pole, 52.6 feet east of broken iron post.

N.W. Cliff Peoa N. 47° E. magnetic.

Lowest notch Vatia pass N. 141.2° W. magnetic.

Beacon on Goat Island reef N. 21° W. magnetic.

Red Buoy (Grampus Rock) N. 75° E. magnetic.

Inner end Goat Island N. 31° W. magnetic.

See plate 1, figure 1,

At 900-foot point on this line a 2-inch iron pipe was driven into reef, filled with concrete, and a mound of cement built about its base. The squares along this line were laid out using the line as their east side, *i. e.*, to west of base line.

Line 2: Extends from sea wall on north side of road to Goat Island at point under east end of north handrail of inshore bridge, N. 27° W. 295 feet to edge of reef.

North end of old rock pier extending north from Goat Island bears N. 41° E. magnetic and northeast corner of dock at government house bears N. 74° W. magnetic.

Heavy galvanized spikes mark 100-foot, 200-foot and 265-foot points, the latter on a big Porites head, along this line. The four corners of a 25-foot square laid out from 200 to 225 feet on this line are also marked by galvanized spikes. All squares laid off on east side of this line.

Line 3: Laid out along east side of landing at Government house. Wall at low-tide mark forming one side of each square.

Line 4: Extends from sea wall at a point 400 feet west from cement walk at inshore end of landing at Government house. Northwest in line with southwest corner of brotherhood school on north side of harbor 125 feet to end of reef. Marked at 100-foot and 175-foot points by galvanized spikes. Squares laid off to east of base line.

Line 5: Same point of origin as line 1. Extends N. 16° W. 615 feet to edge of reef. Marked at 500-foot point by a concrete-filled 1.5 inch pipe bedded in reef. This pipe extends about 1.5 feet above low-tide mark. Squares were laid off to east of this line.

The distribution of the Alcyonaria along the several lines will be given in the following tables and graphs.

Table 4—*Distribution of Alcyonaria along line 1*

(See text-figs. 1 and 2)

Square	Distance from shore	Square feet covered by Alcyonaria	Character of bottom
1	0 to 25	0	Sandy
2	25 to 50	0	Sandy
3	50 to 75	0	Sandy
4	75 to 100	0	Sandy
5	100 to 125	0	Sandy
6	125 to 150	0	Sandy, Porites heads
7	150 to 175	0	
8	175 to 200	10	Sand, dead Porites
9	200 to 225	13	Sand, dead Porites heads
10	225 to 250	40	Rocky
11	250 to 275	18	Nearly dead Porites and spicules
12	275 to 300	17	Porites and spicule
13	300 to 325	2	
14	325 to 350	25	
15	350 to 375	125	Rock
16	375 to 400	25	Some fairly large heads of living Porites
17	400 to 425	95	
18	425 to 450	100	
19	450 to 475	10	
20	475 to 500	75	Very rough bottom composed of dead Porites
21	500 to 525	50	
22	525 to 550	600	
23	550 to 575	500	
24	575 to 600	30	Heads and rocks formed from Alcyonarian spicules, mostly the latter
25	600 to 625	20	
26	625 to 650	4	
27	650 to 675	30	
28	675 to 700	160	
29	700 to 725	450	
30	725 to 750	300	
31	750 to 775	100	
32	775 to 800	400	
33	800 to 825	4	
34	825 to 850	1	
35	850 to 875	8	Lithothamnium ridge, practically bare of all other organisms except a few encrusting acroporas
36	875 to 900	0	
37	900 to 925	0	
38	925 to 945	0	

Table 5—*Distribution of Alcyonaria along line 2*

(See text-figs. 3 and 4)

Square	Distance from shore	Square feet covered by Alcyonaria	Character of bottom
1	0 to 25	2	Almost entirely covered with sand washed through under bridge from flat on north bank Utelei cove.
2	25 to 50	3	
3	50 to 75	0	
4	75 to 100	0	
5	100 to 125	8	
6	125 to 150	50	Several Porites heads
7	150 to 175	200	Alcyonarian rock
8	175 to 200	400	
9	200 to 225	100	
10	225 to 250	30	Dead Porites
11	250 to 275	80	Branching Porites
12	275 to 295	25	Many Acroporas

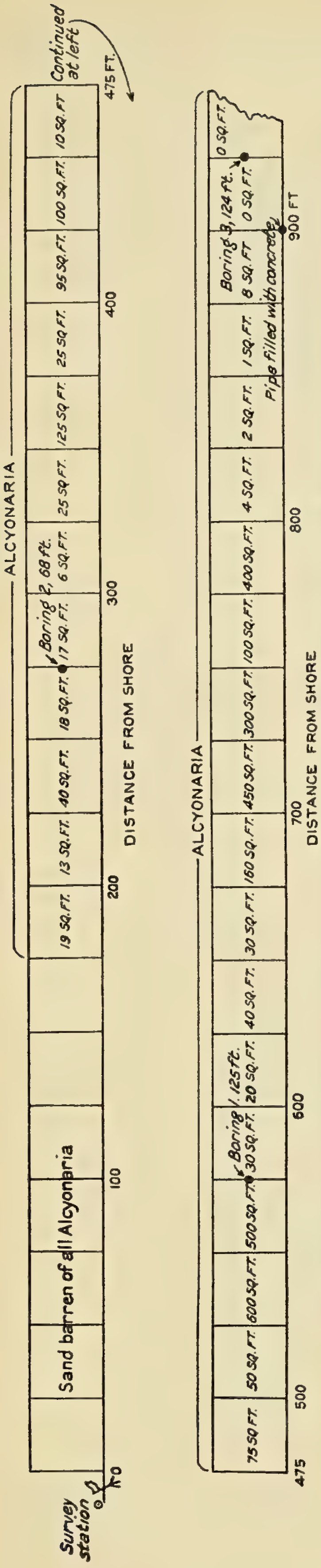

Fig. 1—Chart showing proportion of surface of reef covered by living Alcyonaria in the series of 25-foot squares along line 1.

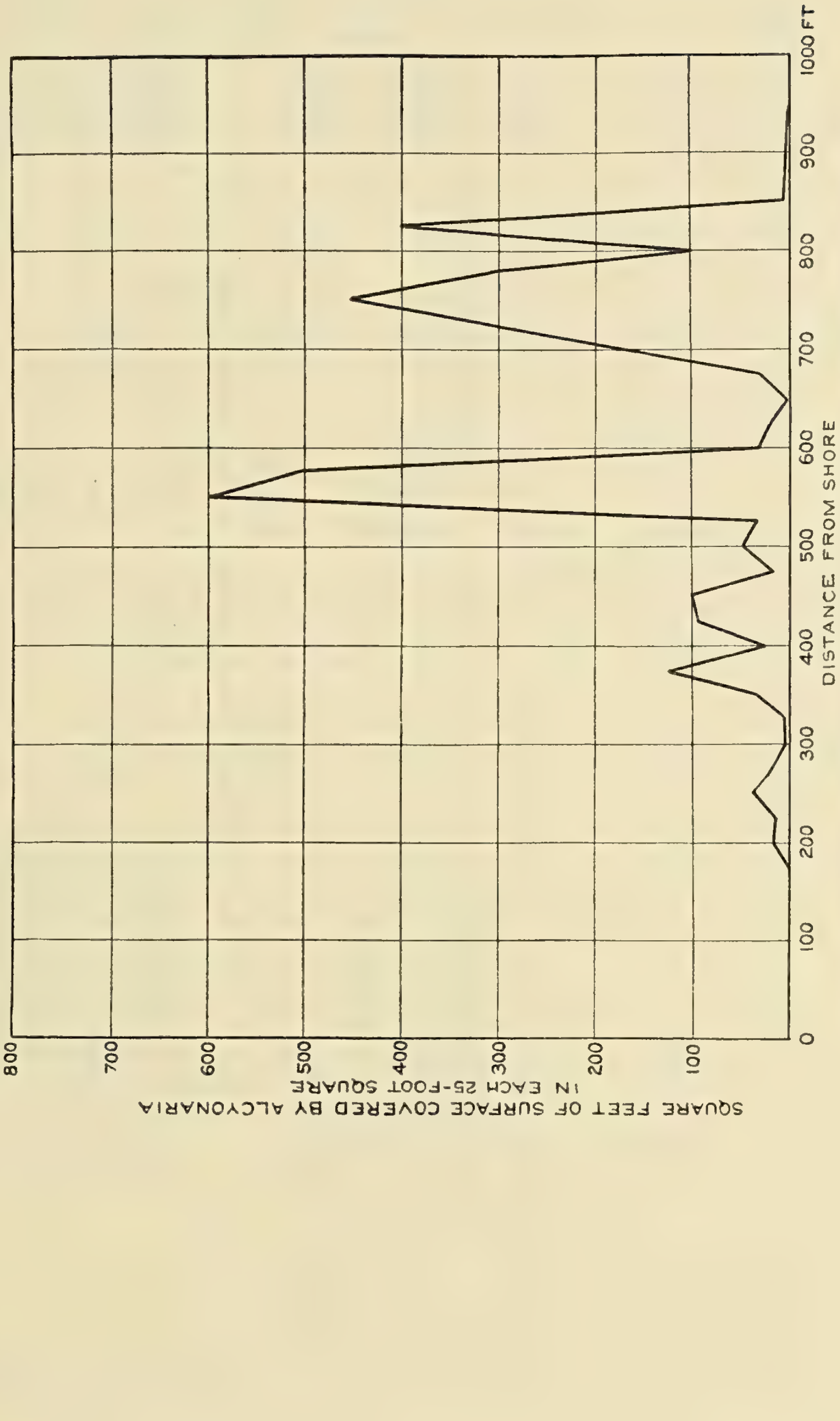

Fig. 2—Graph showing distribution of living Alcyonaria in series of 25-foot squares along line 1.

TABLE 6—*Distribution of Alcyonaria along line 3*

(See text-figs. 5 and 6)

Square	Distance from shore	Square feet covered by Alcyonaria	Character of bottom
1	0 to 25	1 specimen	Sand, volcanic rock
2	25 to 50	20	Sand, volcanic rock
3	50 to 75	40	Porites (branching)
4	75 to 100	200	Dead coral, sand
5	100 to 150	300	Sand, Porites (branching)
6	150 to 200	80	Porites, large masses

TABLE 7—*Distribution of Alcyonaria along Line 4*[1]

(See text-figs. 7 and 8)

Square	Distance from shore	Square feet covered by Alcyonaria	Character of bottom
1	0 to 25	75	Large Alcyonarian heads. Branching Porites
2	25 to 50	250	
3	50 to 75	15	
4	75 to 100	20	
5	100 to 125	400	

[1] Present shore line is formed by a fill about 100 feet wide.

TABLE 8—*Distribution of Alcyonaria along Line 5*

(See text-figs. 9, 10, 11)

Square	Distance from shore	Square feet covered by Alcyonaria	Character of bottom
1	0 to 25	0	Sand Porites heads
2	25 to 50	0	Sand Porites heads
3	50 to 75	0	Sand Porites heads
4	75 to 100	0	Sand Porites heads
5	100 to 125	0	Sand Porites heads
6	125 to 150	0	Sand Porites heads
7	150 to 175	1 specimen	Sand Porites heads
8	175 to 200	0	Sand Porites heads
9	200 to 225	0	Sand Porites heads
10	225 to 250	0	Sand Porites heads
11	250 to 275	200	Rough broken bottom Porites heads and rock of Alcyonarian spicules
12	275 to 300	400	
13	300 to 325	150	
14	325 to 350	300	
15	350 to 375	450	
16	375 to 400	200	
17	400 to 425	400	
18	425 to 450	200	
19	450 to 475	150	
20	475 to 500	300	
21	500 to 525	550	
22	525 to 550	300	
23	550 to 575	100	Lithothamnium ridge
24	575 to 600	20	
25	600 to 615	2	

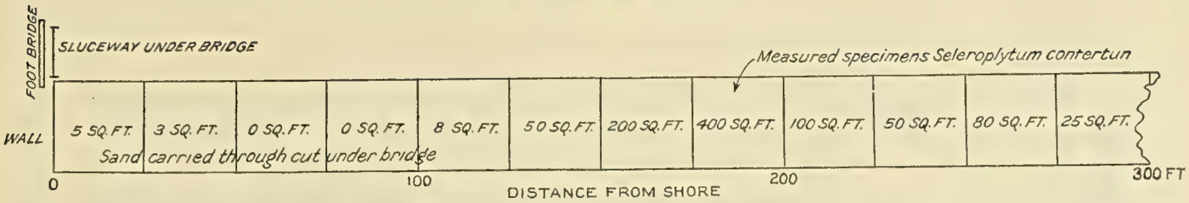

FIG. 3—Chart showing proportion of surface of the reef covered by living Alcyonaria in series of 25-foot squares along line 2.

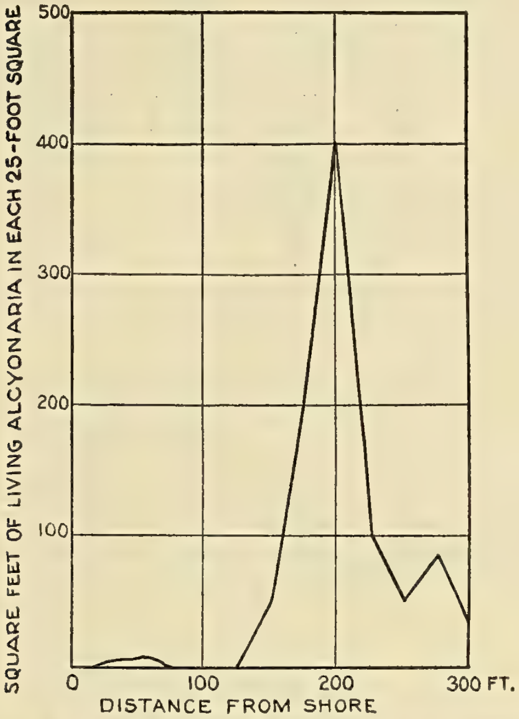

FIG. 4—Graph showing distribution of living Alcyonaria in series of 25-foot squares along line 2.

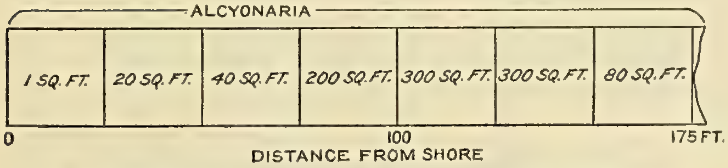

FIG. 5—Chart showing proportion of surface of reef covered by living Alcyonaria in series of 25-foot squares along line 3.

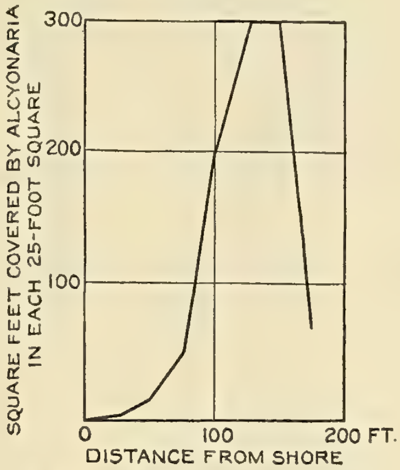

FIG. 6—Graph showing distribution of living Alcyonaria in series of 25-foot squares along line 3.

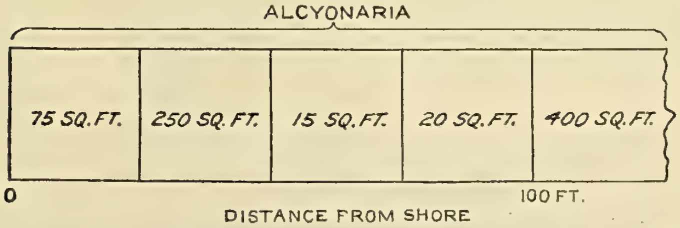

FIG. 7—Chart showing proportion of surface of reef covered by living Alcyonaria in series of 25-foot squares along line 4.

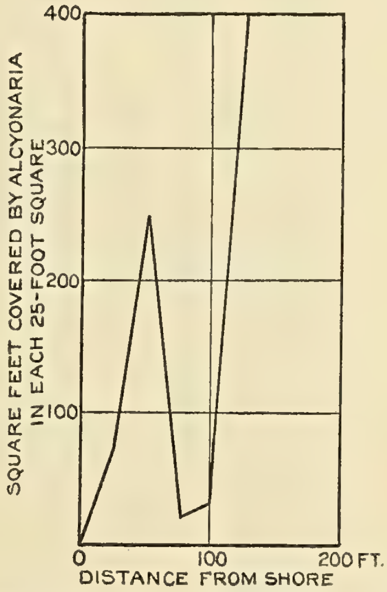

FIG. 8—Graph showing distribution of living Alcyonaria in series of 25-foot squares along line 4.

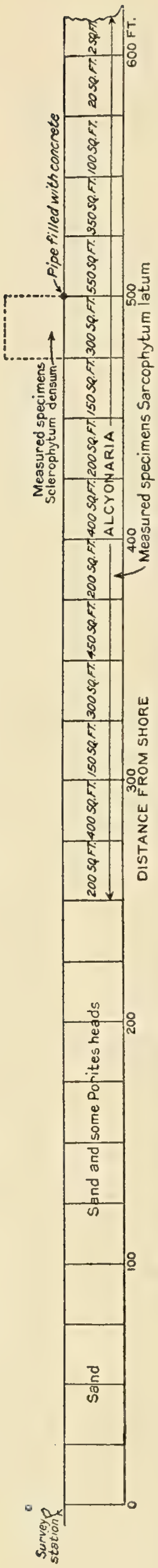

Fig. 9—Chart showing proportion of surface of reef covered by living Alcyonaria in series of 25-foot squares along line 5.

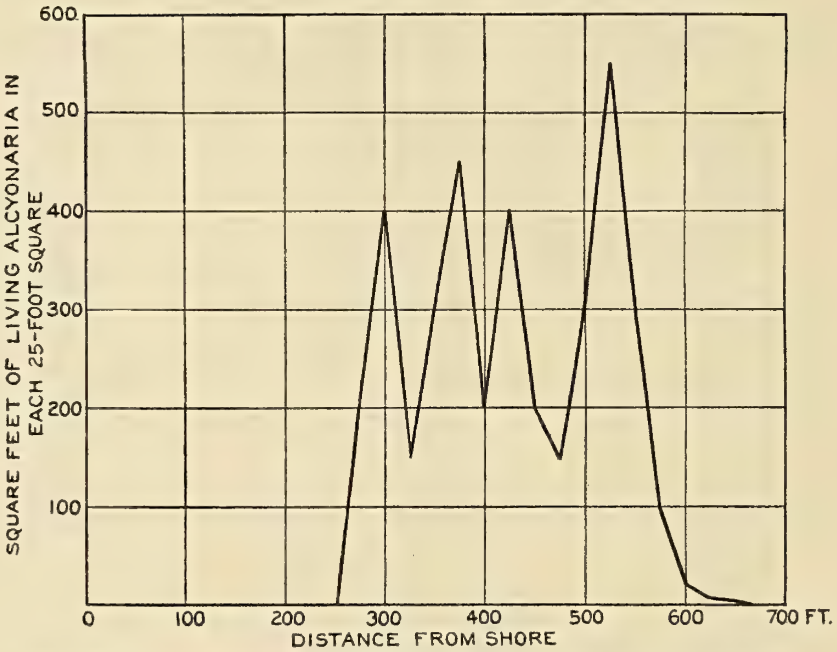

Fig. 10—Graph showing distribution of living Alcyonaria in series of 25-foot squares along line 5.

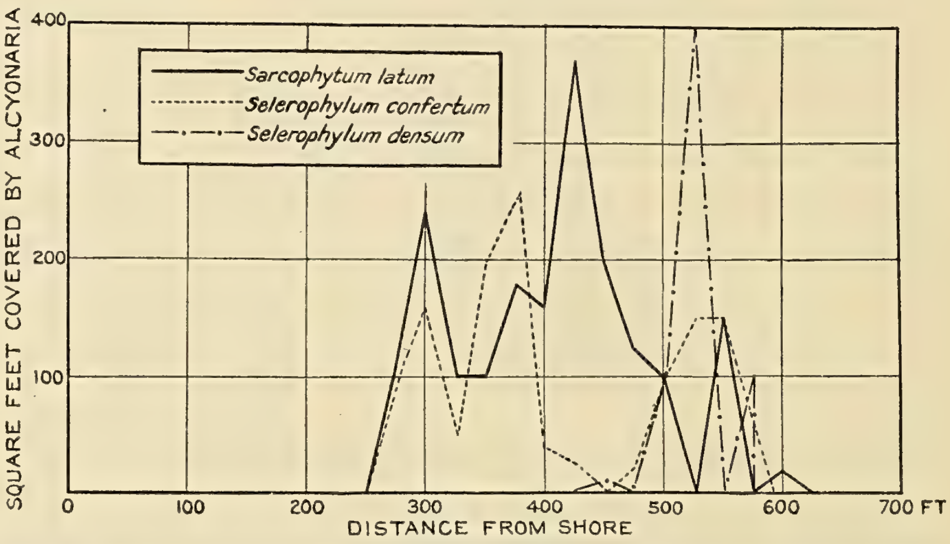

Fig. 11—Graph showing distribution of three important species of Alcyonaria in series of 25-foot squares along line 5.

GROWTH RATES OF SAMOAN ALCYONARIA

In the following tables in which are shown the growth rates of a number of specimens of the four important species of Alcyonaria of the Samoan reefs, the number of the specimen, its location in the square, by measurements from two sides of the square, and its dimensions (1) along its long axis, (2) along its short axis, and (3) its perimeter at the base are given in the first three columns. Since the specimens were all left undisturbed on the reef, the only result of their growth that could be recorded as of importance in the ecology of the reef was the increase in the area covered by each one. Consequently in the fourth and fifth columns are given the ratio to the original area of the area attained after fifteen months and three years, respectively.

TABLE 9—*Measurements of S. densum, April 6, 1917, on a square laid out to west of line 5 from 475 to 500 feet*

(See text-fig. 12)

Specimen No.	Location in square	Dimensions[1]	Relative area after 15 mos.	Relative area after 3 yrs.
1	N.E. corner of square under east line	20″ × 8.5″ x 3′ .3″	Lost	
2	11″ from E. base, 7.5″ from N. base	9″ × 5.5″ × 22″	Lost	
3	4.7′ diagonal from 500 ft. stake 2.8′ N. base	5″ × 3″ × 11.75″	1:1.412	1:2.19
4	Large colony around S.E. side. Porites head 3.7′ from E. base, 1.6′ N. base	38″ long × 15″ N. end 38″ long × 19″ middle 38″ long × 11″ S. end	1:2.17	1:3.17
5	Large mass composed of several separate pieces. 5.9′ from E. base, 4″ N. base.	7′ E & W × 3′ N & S × 14.9′	1:1.12	1:1.18
6	14.2′ E. base, 16.5′ N. base	3″ × 2.5″ × 6″	1:3.13	1:4.62
6a	Under N. line 14.65′ E. base	11″ × 4″ × 22″	1:1.09	1:2.18
7	Almost touches 6a on W. side of former	4.5″ × 4″ × 11.5″	1:1.34	1:5.95
8	17.8′ E. base 22″ N. base	6″ × 4″ × 14″	1:1.30	1:6.53
9	21.8′ E. base 4″ N. base (see sketch)	12″ × 4″ × 26″ middle 12″ × 8″ × 26″ south 12″ × 6″ × 26″ north	1:1.388	1:2.73
10	Under N. line 22.3′ E. base	2″ × 7″ × 21.25″ widest 2″ × 2.5″ × 21.25″ narrows	1:1.26	1:1.39
11	24.2′ E. base (under W. line) 24′ N. base	17″ × 12″ × 3′8″	1:1.19	1:2.28
12	22.6′ E. base 30″ N. base smallest of two 12 and 13	1″ × 2″ × 4″	Lost	
13	Touches 12	3.75″ × 3″ × 9″	1:1.372	1:2.86
14	21.1′ E. base 3′ 10″ N. base	9″ × 6.5″ × 21″	1:1.765	1:2.82
15	20.7′ E. base 5′3″ N. base	5″ × 4″ × 11.5″	1:1.60	1:3.42
16	21.55′ E. base 6′2″ N. base	4″ × 4″ × 11″	1:1.66	1:4.37
17	Large crescentric mass 5.6′ from E. base on a Porites head; 7.0′ from N. base (south of cross-line)	36″ × 19″ × 79″	1:1.632	1:2.37
18	Small specimen on top of Porites head, 7.9′ N. base, 13.3′ E. base	9″ × 7″ × 26″	1:1.238	1:3.37
19	14.9′ E. base, 9.2′ N. base	2″ × 2″ × 5″	1:3.59	1:4.73
20	8.1′ N. base, 21.6′ E. base	6.5″ × 4″ × 15″	1:1.685	1:6.86
21	22.25′ E. base, 7.2′ N. base	11.5″ × 7″ × 26″	1:1.23	1:1.81
22	23.3′ E. base, 7.6′ N. base	12″ × 8″ × 27.5″ west 12″ × 4″ × 27.5″ east	1:1.10	Lost
23	23.3′ E. base, 8.25′ N. base	2″ × 3″ × 8″	1:1.243	1:2.21
24	23.4′ E. base, 8.7′ N. base	9″ × 9″ × 27.5″	1:1.17	1:2.21
25	Under west line 8.7′ N. base	9″ × 7.5″ × 21″	1:2.05	1:14.10

[1] Dimensions are given along long axis, short axis and perimeter at base.

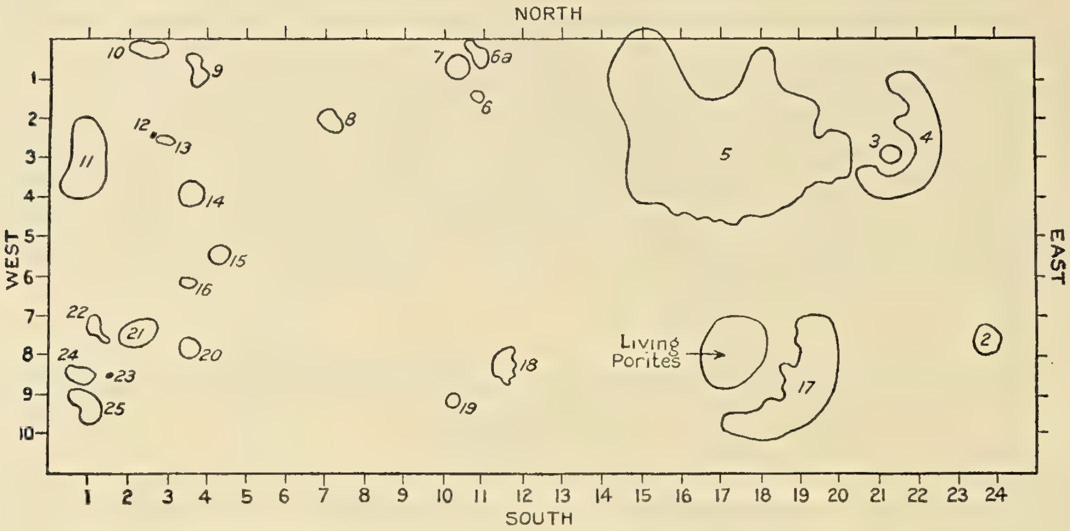

FIG. 12—Chart showing location within a 25-foot square on line 5 of specimens of *Sclerophytum densum* the measurements of which are recorded in table 9.

TABLE 10—*Measurements of S. latum on a square laid out 375 to 400 feet on Line 5—(square east of line)*

Specimen No.	Location in square	Dimension	Areas of base after 15 mos.
1	Under N. base 22.5′ W. base	14″ × 10″ × 27″	1:5.74
2	Under N. base 22′ W. base	7.5″ × 6.5″	1:6.8
3	13.1′ W. base 3′5″ N. base	4.5″ × 3″ × 7″	1:4.61
4	9.9′ W. base 2′7″ N. base	12″ × 9″ × 27″	1:21
5	Touches No. 4 on south	11″ × 9″ × 25″	1:1.26
6	7.3′ W. base 5.1 N. base	8″ × 7″ × 14″	1:2.24
7	4.4′ N. base 2.85′ W. base	10″ × 8″ × 18″	1:2.37
8	5.5′ N. base 11.0′ W. base	15″ × 8″ × 24″	1:1.405
9	5.9′ N. base 22″ W. base	13″ × 8.5″ × 28″	1:1.52
10	6.6′ N. base 6″ W. base (touches No. 11)	11″ × 9″ × 23″	1:2.18
11	6.3′ N. base Under W. line	15.5″ × 10″ × 29″	1:1.25
12	9.3′ N. base 3′ W. base	18″ × 12.5″ × 27	1:1.185
13	9.8′ N. base 5.95′ W. base	4.5″ × 4″ × 7″	1:10.7
14	18.2′ W. base 7.5′ N. base	2.5″ × 1.5″ × 3.5″	Dead
15	19.6′ W. base 12.5″ N. base	4″ × 3″ × 7″	1:5.51
16	19.9′ W. base 12″ N. base	3″ × 2″ × 4.5″	1:11.9
17	20.1′ W. base 22″ N. base	2″ × 2″ × 4″	1:8.12
18	19.9′ W. base 29″ N. base	3″ × 1.5″ × 4″	1:4.44
19	Extreme S. E. corner	6.75″ × 7″ × 12.5″	Lost
20	22.4′ W. base	6″ × 6″ × 11.5″	
21	19.8′ W. base 6″ S. base	4.5″ × 4.5″ × 9″	
22	6.9′ W. base. Under S. line	12.5″ × 9″ × 19.5″	1:2.76
23	Extreme S. W. corner	19″ × 12.5″ × 39″	1:1.15
24	23″ W. base 17.3′ N. base	19″ × 13″ × 40″	1:1.76
25	16.3″ N. base 25″ W. base	13″ × 7.5″ × 23″	1:1.18

TABLE 11—*Measurements of S. confertum on Square No. 8 Line 2, March 24, 1917*

(See text-fig. 13.)

Specimen No.	Location in square	Dimensions	Areas of base after 15 mos.
1	Solid colony 30″ N. 23″ E.	7.25″ × 6.5″ × 15.5″	Lost
2	Irregular broken colony 3.75″ W. base 2′ N. spike 1a.	28″ × 26″ × 81″	Lost
3	Lead colored, under S. line 7″ W. spike 1a	35 mm. × 45 mm. × 125 mm.	1:16.00
4	Lead colored 13.5″ S. base; spike 1a	26 mm. × 7″ × 6″ × 13.5″	Lost
5	Small lead colored in 1a 16″ N.W. spike 1a 10″ S.E. corner No. 2	53 mm. × 36 mm. × 68 mm.	1:27.9
6	Large brown colony in 1c, S. edge under line S.W. corner 11″ spike 1b	22″ × 14″ × 52.5″	Separated into several distinct parts
7	Very small lead-colored colony in 1d straight N. from Acropora, 39″ S. base 49″ spike 1c, 64″ spike 1d	30 mm. × 14 mm. × 53 mm.	1:102.00
8	Lead colored in 1c. 34″ stake 1d, 26″ stake 2d, 12″ E. base	10″ × 10″ × 16.5″	1:3.24
9	Lead colored in 1d, 25″ E. base 12″ N. cross-line	6″ × 4″ × 10.5″	1:1.81
10	Lead colored. S. W. corner 2a	3.5″ × 3″	
11	In 2a 5.3″ from a pipe 2a, 5.5′. 3a	11″ × 13″ × 30″	1:2.0.81
12	On S. line 2a, 5.4′ spike 2a	13″ × 9″ × 26″	1:1.065
13	(In 2a) 7.9″ E. base, 1.6′ N. cross-line	5.5″ × 3″ × 9″	1:2.43
14	Very small head about which a space was cleared 10.3′ E. base, 2.2′. N. cross-line	1.5″ × 2″ × 5″	1:16.00
15	Begins 1.0′ from No. 14:0.85′ S. base 1.2 N	33″ × 22″ × 72″	Broken up
16	16.2′ E. base 0.25′ N. cross-line	12″ × 9″ × 26″	1:1.27
17	18.5′ E. base under S. cross-line	7″ × 5″ × 18″	1:7.15
18	20.5′ E. base 1.0′ S. cross-line	5″ × 3″ × 7″	1:18.58
19	Large mass just N.W. No. 18 about 2″	4′5″ × (25″ west, 24″ east, 11″ middle, × 117″)	Broken into many units
20	21.45′ E. base 0.2′ N. line	3″ × 3″ × 9″	1:4.32
21	Against W. base .6′ N. line	9″ × 11″ × 22″	1:2.077
22	3.4′ E. base 1.9′ N. line	4″ × 6″ × 5.5″	Lost
23	3.8′ E. base 2.0′ N. line	6″ × 5″ × 15″	Lost
24	Large brown head 5.25′ E. base 1.1′ N. line	16″ × 18″ × 44″	1:1.08
25	In 2a cleared up around it. 7.9′ E. base 1.1′ north line	1.5″ × 1.5″ × 3.5″	1:9.96
26	6.3′ E. base 3.7′ N. line	24″ × (17″ W., 9″ E., 4″ N.W.)	Too badly broken up to measure
27	10.5′ E. base 3.0′ N. line	17″ × 13″ × 32″	1:3.78
28	19.7′ E. base 3.9′ N. line	10″ × 7″ × 23″	Lost
29	Old head partially dead. In 4 & 5:1.7 E. base	1.9″ S. end 36″ × 27″ widest × 7′11.5″	Broken up so its identity was lost
30	Extreme N.W. corner	(19″ N.) 20″ × 16.5″ × 51″	Lost
31	5.7′ E. base 0.9′ N. base, dead in center	27″ × 26″ × 72″	1:1.03
32	Large head dead center (line W. of No. 29) (on line between squares 4 and 5) Cavity in center is 13.5″ E. and W., 15.5″ N. and S.	3′8″ × 3′9″ × 9′10″	1:1.17
33	Large head on N. base 10′ W. base	51″ × 27″ middle 16″ east 17″ west } × 114″	Broken into several individuals
34	On N. line 15.4′ E. base	27″ × 22″ × 72″	1:3.78
35	Under line between 4 and 5 20.5′ E. line	31″ × 41″ × 98″	1:1.49
36	Just E. square 4. E. end under the E. base line of large square	11.6″ in circumference	1:1.355

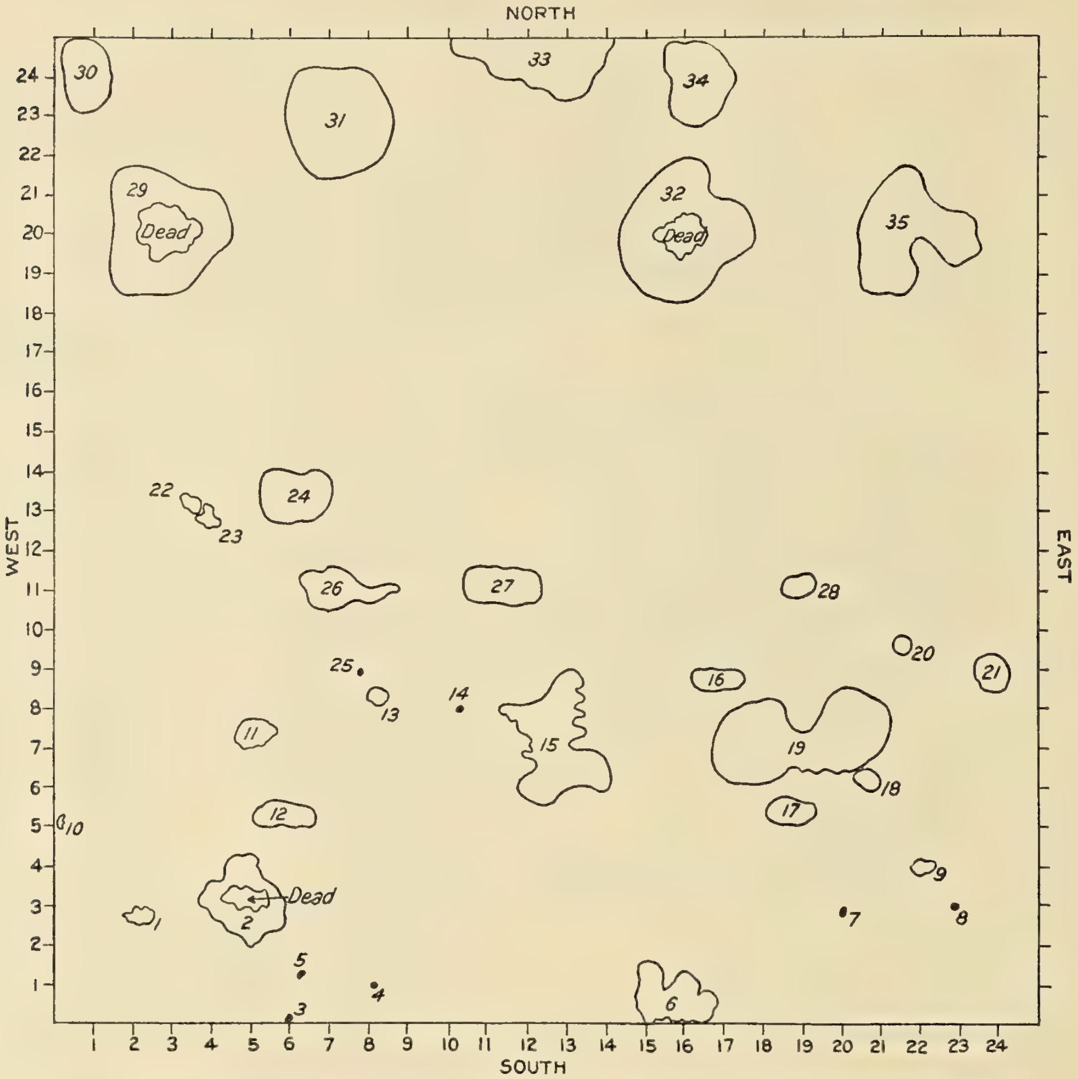

FIG. 13—Chart showing location within a 25-foot square on line 2 of specimens of *Sclerophytum confertum* the measurements which are recorded in table 11.

TABLE 12—*Measurements of N. flexile on Line 3 along Government landing*
(All between two galvanized spikes driven into the stone wall along the dock)

Specimen No.	Location in square	Dimensions	Relative area after 15 months
1	Near a coral head about 1′ from S. spike	4″ × 3″ × 6″ × 3″ high	Lost
2	4 specimens (No. 2-No. 8) in two lines along sea wall. No. 2 is farthest to left on upper line	13″ × 9″ × 17″ × 5.5″	Separated into several individuals
3	Next to right (N.)	8″ × 5″ × 6.5″ × 5″	1:6.66
4	Next to right 3 divisions	11″ × 9″ × 12″ × 5″ (upper stalk)	1:11.85
5	Southernmost on lower line	10″ × 7″ × 9.5″ × 5″	1:3.92
6	Next north	13″ × 8″ × 10″ × 9″	Divided into two
7	Next north	6″ × 5″ × 7″ × 9″	1:7.86
8	Last one north	6″ × 5″ × 9″ × 4″	1:6.36

TABLE 12—*Measurements of N. flexile on Line 3 along Government landing*—(Continued)

Specimen No.	Location in square	Dimensions	Relative area after 15 months
9	9 to 16 in a clump growing out from wall starting 12′ from S. stake	10″ × 9″ × 8″ × 6″	1:2.81
10	Just north of No. 9	9″ × 6″ × 7″ × 6″	1:5.55
11	East of No. 10	7″ × 6″ × 9″ × 6″	1:1.56
12	North of No 11.	14″ × 7″ × 14″ × 6″	1:10.6
13	West of No. 11	15″ × 10″ × 18″ × 8″	1:5.25
14	Whole N.E. part of mass	20″ × 22″ × 27″ × 9″	1:6.6
15	South of No. 14	7″ × 6″ × 9″ × 6″	1:4.07
16	Extreme east of cluster (17 to 25 in cluster about 3′ from N. spike at inner end (No. 17) extends opposite N. spike)	7″ × 7″ × 10″ × 5″	
17	Specimen on coral block	6″ × 6″ × 6″ × 6″	Lost
18	N. from 17	5″ × 5″ × 6.5″ ×..	1:4.02
19	20-21 form a triangle N. of general mass	9″ × 9″ × 8″ × 6″	1:2.75
20	Nearest wall	5″ × 7″ × 6″ × 6″	1:2.75
21	Straight out from 20	7″ × 5″ × 6″ × 6″	1:8.05
22	N.E. innermost of bunch	4.5″ × 3″ × 5″ × 3″	1:16.02
23	Easternmost	7″ × 5″ × 6″ × 6″	1:2.275
24	Just south of 23	7″ × 7″ × 8″ × 8″	1:1.00 Divided nearly to base
25	Next N.W. of 24	9″ × 6″ × 8″ × 8″	1:5.12
26	West of 25	16″ × 9″ × 18.5″ × 8″	Lost
27	Westernmost of dense cluster	11″ × 7″ × 14.5″ × 6″	1:2.75

When these specimens were measured in 1918, 26 individuals not there in 1917 were found on the area of bottom on which were located the 27 for which measurements had been recorded the year before. The dimensions of 10 of these individuals, not more than 15 months old, were:—

1a	4″ × 2.5″ × 4″ × 5″ high	6a	5″ × 5″ × 6″ × 7″ high
2a	5″ × 5″ × 2.5″ × 7″	7a	6″ × 5″ × 5″ × 8″
3a	4″ × 5″ × 6″ × 8″	8a	4″ × 6″ × 5″ × 7″
4a	3.5″ × 3″ × 4″ × 6″	9a	5″ × 5″ × 4″ × 9″
5a	6″ × 5″ × 6″ × 8″	10a	7″ × 4″ × 5″ × 8″

From evidence furnished by specimens 2, 6 and 25, it would appear most probable that the greater number of these new individuals had arisen by the process of longitudinal division of some of the older colonies and not from the development of planulæ which had settled among the older specimens. Relatively large clusters, such as that made up of individuals 17 to 25, inclusive, have quite possibly arisen by the repeated fission of a single original specimen and its asexual descendents over a considerable period. The fact that the height of the new individuals was practically the same as the older ones, while their other dimensions were considerably less, adds weight to this conclusion.

As a check on the growth under more carefully determined conditions, 25 specimens of *S. confertum* were obtained by breaking the skeletal mass below growing "heads" of branching colonies of this species. These were put out near the border of the Utelei south reef at its northwest corner. The location selected was near to areas where *S. confertum* was abundant on a bottom where shifting coral sand was the chief constituent. The barren spicule skeletons were embedded in a mass of cement 7′ 6″ by 5′ 1″ which extended down to a solid foundation below the shifting sand. The long axis of this "bed" was approximately north and south. The position of the specimens in the plot is shown in text-figure 13.

Since this entire reef area is devoid of any living stony corals and no Lithothamnium ridge is formed so far in from the entrance to the cove and since none of the other species of Alcyonaria were found near by, it would appear that of the common reef-forming organisms *S. confertum* alone was able to flourish under the environmental conditions existing here where the greater part of the coral sand found on the inner portion of the surface of the Utelei south reef spills over into the deep channel of the cove.

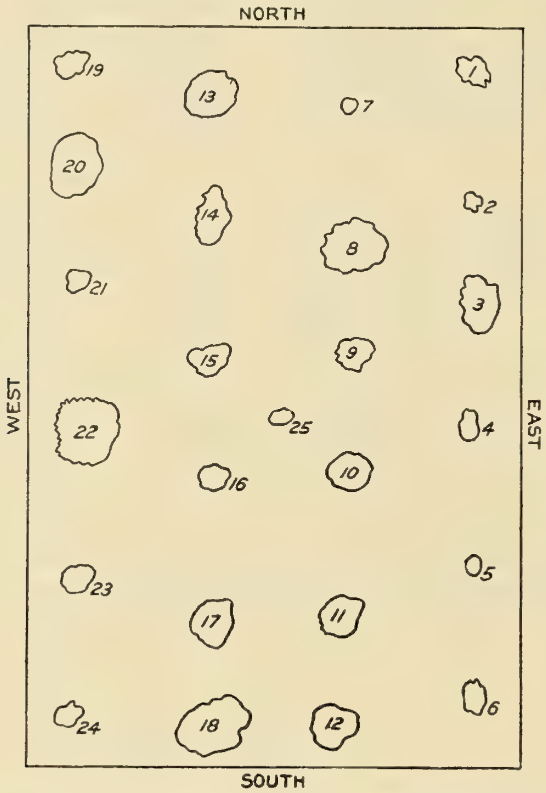

Fig. 14—Chart showing location on the cement bed, made on the NW. corner of the Utelei south reef, of specimens of *Sclerophytum confertum* planted out in Aug. 1918, the measurements of which are recorded in tables 13 and 14.

When this planting was made it was thought probable that the existence of a naked mass of spicule rock below the living tissue in all old colonies of *S. confertum* might be due to the destruction of the organic portion by abrasion by the shifting sand and that the perpetuation of the colony was possible only because some of its branches had been able to keep their apices above the sand. When the planting was examined after one year (by Dr. Mayer) the living tissue had in all instances grown down over the bare skeleton to the cement, although varying amounts of sand, up to 3 inches in depth, were found over the surface. Under natural conditions then, some cause other than such abrasion must be accountable for the death of the lower portions of the colony. The presence on the reefs of the inner harbor of large masses of barren spicule rock capped by living colonies of *S. confertum,* which extend from the general reef surface to approximately spring tide low-water level, a distance of some 3 feet, gives the impression that most vigorous growth takes place only at the apex of a colony, and this fact, in addition to the observed death of irregular portions of the tissues over the central areas of large colonies would in time bring about the formation of a branching and anastamosing skeletal framework below the growing portion of any older colony.

The dimensions of the 25 specimens on the "bed" at the time of their planting and again at the end of approximately one year are given in the following tables.

TABLE 13—*Measurements of S. confertum cemented on N.W. corner of South Reef at Utelei, August 2, 1918*
(See text-fig. 14)

Specimen No.	Height of living tissue above concrete	Perimeter base of colony	Height of colony	Perimeter old skeleton	Long axis	Short axis
1	36	227	130	245	132	101
2	22	260	80	295	127	92
3	55	440	232	394	250	232
4	12	303	150	308	190	151
5	Flush	320	60	0	180	70
6	5	112	65	. . .	60	55
7	Flush	220	100	0	120	100
8	40	268	160	250	170	140
9	Flush	390	150	0	230	177
10	Flush	405	180	0	225	100
11	Flush	206	122	0	155	106
12	Flush	273	114	0	130	109
13	14	293	90	281	162	133
14	Flush	400	125	14 mm. between colonies		
15	Flush	280	150	0	100	170
16	Flush	280	100	0	205	110
17	Flush	365	95	0	148	112
18	Flush	350	143	0	155	153
19	Flush	180	80	0	212	90
20	Flush	384	143	0	153	160
21	25	195	120	157	240	84
22	90	360	196	295	95	177
23	45	235	130	220	230	130
24	Flush	116	62	134	185	55
25	22	153	78	. . .	80	48
					102	

TABLE 14—*Measurements of the same specimens on August 20, 1919*[1]

Specimen No.	Perimeter of base of colony	Height above concrete	Long axis	Short axis
1	258	148	212	169
2	164	90	120	78
3	405	246	328	265
4	361	160	320	194
5	176	66	110	110
6	149	65	104	53
7	225	115	206	138
8	275	184	327	214
9	373	162	271	204
10	390	197	341	193
11	239	131	252	185
12	313	120	190	190
13	354	116	250	174
14	463	147	308	155
15	306	180	330	209
16	320	115	181	115
17	511	110	290	195
18	365	173	353	252
19	307	105	190	129
20	493	176	340	218
21	191	140	195	154
22	327	227	310	239
23	291	164	246	200
24	193	75	120	85
25	160	83	135	48

[1] 1919 measurements by Dr. Mayor.

PART II

THE STRUCTURE OF THE CORAL REEFS IN PAGO PAGO HARBOR

In order to determine the origin of the limestone making up the mass of the Utelei reefs on the surface of which the studies described in the previous section were made, the character (origin) of the surface rock was studied in detail along line No. 1. This was done by digging up the entire reef surface over an area of one square yard at intervals of 10 feet, thus giving 94 stations from which samples were taken. The material obtained was for the most part discarded at once after an examination of its structure and component skeletal remains had been made immediately upon its separation from the underlying limestone. Other, especially more doubtful, material was taken to the laboratory for more thorough examination.

For the distance of 175 feet from the shore, which at this point consisted of a retaining wall of basalt erected in the building of a roadway around a sharp point, the bottom was composed of loose coral sand, while the only living coral organisms were a few scattered heads of massive *Porites*. A much larger number of dead skeletal masses of the same *Porites* were located over these areas, some of them extending a few inches above the general level of the reef surface. On working over the shifting material, down to the solid substratum, a number of *Sclerophytum confertum* spicule masses, of the character illustrated in figures 18, 19, plate 7, were encountered both in the sand and in the superficial layers of the solid reef rocks. The presence of these structures and that of the *Porites*, both living specimens and skeletal remains, would indicate that within comparatively recent times conditions had been much more favorable for the growth of these organisms than they are at present. At a number of stations across this sandy area the hard surface was dug up to a depth of 2 feet, the principal component elements being found to be the massive *Porites*, a branching *Porites* (*P. andrewsii*) and *Sclerophytum confertum* spicule rock, either in the form of the nodules previously mentioned as occurring in the shifting sand or as larger branched masses. All interstices among the more solid rock were naturally filled with calcareous sand, usually unsolidified so that it quickly washed away as the surface material was handled. At present the currents across this barren area, at all times except at high tide, set to the westward and are, under most conditions, of sufficient strength to move the coarse sand with a noticeable velocity. The configuration of the shore-line and of the reef flat in relation to the axis of the harbor is such that the greater part of the smaller loose particles arising on the entire reef area from the Fagaalu north reef and that off Blunts Point are here carried along the reef moat to spill over into the deeper water at the northwest corner of the Utelei south reef. The abrasion caused by this current-borne material, as especially stressed by Crossland ('27-'28), may be sufficient to cause the destruction of the "corals" of both the Madreporarian and Alcyonarian types. That conditions favorable for the growth of reef-building corals occurred until the level of the reef had reached that of the present hard substratum is quite possible since the strength of the currents has been observed to be dependent upon the depth of water over the reef flat as a whole. When the tide has risen one foot above mean low tide there is no longer a marked spilling over into the reef moat and the strong current following the shore line becomes much less rapid.

Beyond the 175-foot point the reef surface was, at all the stations, either covered with living coral-forming organisms, or, where barren of such material, clearly made up of limestone composed of the skeletons of these forms. On 83 of the 94 areas examined, limestone composed of the skeletal remains of *S. confertum* or *S. densum* was recognizable among the loosened material. On areas where the greater part of the surface was covered by living colonies of *S. confertum* (see chart No. 1) practically the entire surface rock was, as would be expected, composed of the cemented spicules of this species.

On the Lithothamnium ridge, which was approximately 60 feet wide at this point, only one specimen of spicule rock was found on the surface, and that at the base of a living colony of *S. densum* living in the breakers at the reef edge. The examination of the more deep-lying material showed that on some of the areas a relatively large proportion of the material removed was composed of spicule rock where the entire surface has been covered with *Porolithon.* The evidence obtained when the boring was made through this material, at 925 feet from shore on line 1 (See Chart No. 1), fully confirmed this observation and showed that at this point, as well as farther shoreward (675 feet) where the surface was carpeted with living *S. confertum,* a large part of the solid reef crust, which extends downward for some 6 feet, was to a great extent composed of *Sclerophytum* spicule rock. In the range between the 175-foot mark and the inner edge of the Lithothamnium ridge on only a single area of those examined, no *Sclerophytum* spicule rock was found in the surface layer of limestone.

Observations at many other points scattered widely over the surface of this reef showed that at least 75 per cent of its surface area is, or has been very recently, covered with living *Sclerophytum* for a period of sufficient length to allow the formation of spicule rock from a small fraction of an inch to several inches in thickness.

RECORDS OF REEFS OTHER THAN THOSE RECORDED IN THE PREVIOUS DISCUSSION

Many other reefs besides those across which lines of squares were laid out to determine the area occupied by Alcyonaria were studied less intensively.

On the reef north of the Utelei entrance upon the inner portions of which there is but a sparse growth of corals, only a very scattered growth of Alcyonaria occurs over the surface of the reef. There is, on the contrary, an almost continuous covering of *Sclerophytum* which extends from low-water mark on the surface of the Lithothamnium ridge down to a depth of 20 feet or more. Along the inner portion of the reef, this depth carries down below the abrupt reef cliff on to the more gradual slope of the foot of the reef where there is a dense growth of loosely branching *Porites,* interspersed with large colonies of *Millepora* resembling in shape and appearance some of the larger cacti, like the *Cereus* of the American desert.

While in many places along this reef edge there are smaller areas barren of either living corals or Alcyonaria, in almost every instance the surface of these areas is found to consist entirely of a layer of rock formed from spicules of *S. densum* making a veneer from 4 to 12 inches in thickness. Whenever stony corals were found in any number along this area, it was observed that every species except a few specimens of the flat mushroom-like *Acropora* were being rapidly overgrown and killed by *Sclerophytum.* This feature of the ecology of the reef edge was especially conspicuous when large specimens of massive *Porites* were involved, as all stages in the overgrowth of the coral could be easily seen.

The reef edge, at a point about two-thirds the distance in from the extremity of the Goat Island flat, was broken down by the use of a small charge of dynamite laid on the upper surface of a small shelf. The material loosened by this small blast consisted almost entirely of rock composed of spicules, with an underlying base of Lithothamnium and coral skeletons. Later a much heavier charge was inserted into a cavern in the reef to a depth of about 4 feet and the entire reef-margin broken up. The upheaval produced by this charge was so great that when the mud had cleared away and the broken material could be examined, most of the fragments were no larger than one's fist and no reliable determination of their former location in the reef could be made. Fragments of rock composed of alcyonarian spicules were intimately mixed with others composed entirely of coral skeletons, while throughout the mass there was a considerable amount of dark grey mud, the constitution of which could not be determined except by chemical analysis. When the finely fragmented material had been cleared away as completely as possible, larger masses from the less broken portions of the reef were pried out and examined without its being found possible finally to determine whether the present surface covering of the reef with rock composed of spicules is an unusual occurrence or if the same condition has been maintained for long periods in the history of this reef.

On many areas over the reefs along the north side of Pago Pago harbor Alcyonaria, especially *S. confertum,* are abundant and form large "heads" composed of rock formed from spicules as on the reefs more carefully studied. On the nearly vertical sides of all the reefs well within the harbor where observations were made, a large portion of the total area was invariably clothed with living Alcyonaria, while the barren areas scattered between or among the masses of living colonies had the appearance of being for the greater part made up of rock composed of spicules.

The chief characteristic of this rock as contrasted with that composed of coral skeleton is the smooth rounded character of the former as contrasted with the much more jagged contour presented by the reef edges formed entirely from stony corals.

In order to obtain as far as possible a comparison between the distribution of the organisms contributing limestone to the present reef surface and that which existed during the formation of the great mass of the reef, a boring was made at a distance of 575 feet from the shore on line No. 1 (see location on map). When the underlying basalt was encountered at a depth of 124 feet, it was thought desirable to make additional borings, not so much to supplement the information obtained regarding the structure and composition of the reef, as for the light that might be thrown by the data from such a series of borings upon the question of the relation of the modern coral reefs to the substratum, especially in reference to the glacial control theory of Daly. With this in view, a boring was made at a distance of 280 feet from shore, and later a third boring on the same line at a point within 20 feet of the reef margin on the Lithothamnium ridge, at a distance of 925 feet from shore.

The conditions encountered, both as regards mechanical difficulties and as indicating the structure of the reef, were so nearly identical for each of the borings that a rather complete account of that first carried out will alone be given. The principal difference between the borings—the depth at which the substratum was encountered in the boring nearest shore—is of importance only as regards the history of the development of the reef in relation to the substratum, and need be referred to in any detail in a later connection only.

LOG OF DRILLING NO. 1 THROUGH THE UTELEI REEF

The drilling machinery employed consisted of a Davis Calyx-Core drill with cutting tools of two sizes, the larger of which would remove a core of 4.5 inches, the smaller a core of 2.5 inches. But, because of the diameter of the only pipe available for a casing for the bore hole, which was necessitated by the character of the material through which we were drilling, the smaller tool alone was used.

In the Davis drill the core barrel above the cutting tool is supplemented by the so-called calyx barrel, a steel pipe of the same diameter as the core barrel and screwed on to the head of the latter so that any material washed up by the force of the stream of water through the hollow drill rods from the point where cutting was taking place would be caught and not, when the water pressure was removed, be allowed to fall back upon the top of the core barrel, where it might either jam this part of the machine when being withdrawn, or be itself ground to small fragments between the wall of the boring and the core barrel.

Because of the generally loose and friable nature of the material found in nearly all except the upper layers of this reef, the rapidly rotating cutting tool with its relatively coarse teeth broke up the coral so that a hole considerably larger than the diameter of the tool was formed, and many of the fragments were consequently washed into the calyx barrel which was usually the most important collector of our specimens.

With the drilling machinery set up on a rough platform about 2 feet above high tide, the first attempt at a boring was begun from the east side of the platform. At this point the reef surface bore very few living corals and was chiefly composed of rock formed from *Sclerophytum confertum* spicules. For a depth of approximately 6 feet the rock was hard and composed of clearly recognizable madrepore skeletons and cemented spicules, through which the drill cut rapidly and of which a nearly continuous core was obtained.

Immediately beneath this hard superficial layer, the reef material was so soft that the cutting tool when rotated sunk of its own weight to a depth of 14 feet. Each time the tool was withdrawn the bore hole filled with sand so quickly that even when the drill was at once lowered again into the hole the sand had to be forced out, and to reach the depth already attained took longer than it had the first time.

Because of the binding of the drilling tools by the loose sand which flowed into the hole from all sides no further progress was possible until a casing was put down, which at the 14-foot level struck a layer so firm that it could not be broken through by driving the casing. After cutting through this dense layer, which was about one foot in thickness, another loose soft layer 4 feet thick was encountered, from which only sand and some fragmentary coral were obtained. The pipe became bent in driving the casing through the dense layer, so that when the cutting tool was again lowered it bound within the casing so firmly that this began to rotate with the drill, and when an attempt was made to withdraw the cutter the casing was withdrawn with it and the two were separated only after long-continued effort. After repeated failures to get down with the drill for any distance below a second dense layer at 19 feet from the surface, drilling was given up. To learn more of the character of the underlying material a ¾-inch pipe through which water was pumped was worked down from the hole already made. By this means it was possible to reach a depth of 46 feet before any obstacle was encountered too resistant to be broken through by using this length of pipe as a hammer when raised for some 4 feet and allowed to drop.

Throughout the extent of this exploratory "sounding" the reef appeared to be made up of loosely disposed rock with a great deal of sand, which when washed away by the stream of water allowed the pipe to advance by its own weight.

After a number of unsuccessful attempts to reach greater depth at the first location, the drilling machinery was turned and another boring started from the west side of the platform. In the upper levels the conditions encountered exactly duplicated those described for the previous attempt. A complete and essentially continuous core was obtained for the first 6 feet. After penetrating this solid layer, the cutting tools sank rapidly to the hard layer at approximately 15 feet below the surface. Profiting by our experience with the previous boring, the water jet was turned on at full strength while penetrating the loose layer, and as a result so little material had accumulated in the core barrel that we were able to penetrate the second dense layer before having to withdraw the drill. When, in driving down a 4-inch steel pipe as a casing, the solid layer at 15 feet depth had been penetrated, the casing advanced easily for some 4 feet, where another dense layer was encountered. This obstruction could not be broken through by light blows on the casing and the cutting tools were again brought into use. After cutting through about 18 inches of solid limestone another loose stratum was found. This layer, through which the cutting tool advanced very rapidly and the casing went steadily with relatively slight resistance, extended to a depth of 46 feet. While the material brought up in the core barrel and calyx barrel indicated that practically the same components entered into the structure of the reef as in the higher levels, the sand particles became progressively finer and adhered to one another much more tenaciously, so that the bore hole did not fill so quickly when the tools were withdrawn. Except for the dense layers encountered at intervals, the only long pieces brought up in the core barrel were sections through heads of the massive *Porites* or masses of *Sclerophytum* spicule rock encountered from time to time in the course of drilling.

At the 46-foot level another hard crust approximately 2 feet in thickness delayed the advance of the casing tube. Beyond this more solid layer no difficulty was encountered for the remainder of the day, during which a depth of 54 feet was attained. Because of heavy rains and mechanical difficulties, due to a jammed coupling in the drill rod, no progress was made throughout the next day. When the drilling mechanism had been put in order once more, progress from this point on was much more rapid, since beyond a depth of 60 feet the lining of the bore hole was no longer necessitated. In one day (July 27) the bore hole was carried from 54 to 105 feet with no interruption in the advance of the cutter, except as was necessary to withdraw the tool in order to take the material from the core barrel.

Throughout this portion of the reef the solid constituent elements consisted of skeletons of *Porites andrewsii*, *Sclerophytum* spicule masses and sections through massive *Porites* skeletons. The material from the interstices between the solid coral was here much changed from the nearly white, coarse and easily movable sand encountered in the upper 45 feet of the reef next below the present surface crust. The original character of the sand grains had become so changed that any external evidence as to the organisms from the skeletons of which they had been derived was not evident on naked eye examination. Indeed, the term coral sand could with propriety no longer be applied to this material since in most of the samples the loose fragments had all assumed a nearly black color; and while in the higher levels below 60 feet it was decidedly plastic, so much very fine material entered into its composition that it might be better described as mud rather than wet sand. At the lower levels the black interstitial material had become solidified between the coral skeletons. Quite commonly the line of demarcation between the original solid coral and the extraneous material deposited between the branches was no longer to be distinguished. At the center of such a mass there would be found a small core of apparently unchanged coral limestone while its peripheral portion had been replaced by, or changed into, a black substance similar in appearance to that extending from one unchanged coral axis to that of a neighboring branch. The mud-like appearance, as well as the color, of the more plastic portions of this substance suggested the possibility that volcanic mud might be a considerable element in its composition, but its almost complete solubility in hydrochloric acid showed that it was practically all limestone, although decidedly changed in appearance.

The difference in the character of the interstitial material in the lower levels of the reef as contrasted with those in the upper 50 feet is well shown by the fact that, although between the time when we had reached a depth of 105 feet and when drilling was undertaken again some 40 hours had elapsed, it was not necessary to spend any time in cleaning out the bore. When first put down, the tool sank freely to the bottom and cutting in new rock began at once. In the upper levels, as previously noted, the sand ran into the bore to an extent sufficient to entirely fill it each time the tools were withdrawn, and any progress after the first passage of the drill through any given section was impossible without the use of a casing.

From the 105-foot level the core consisted of the same black mud-like material with the central axes of relatively unchanged coral branches, but with an increasing proportion insoluble in hydrochloric acid. At the lower depths the "mud" became almost pure laval mud upon which no change was brought about by the addition of acid.

At a depth of 121 feet a solid core 6 inches in length from a *Siphastrea* skeleton was brought up in which the chemical change, so strongly marked in *Porites andrewsii,* was hardly noticeable, so that the specimen appeared in no way different from a section of the same species growing on the present surface of the reef. The evidence from the other material obtained at this level would make it appear likely that this head was entirely surrounded by laval mud and consequently not subjected to the same chemical action as the coral from slightly higher levels.

From the 121-foot level to the final depth of the bore at 124 feet, the last 6 inches of which were represented by a clean-cut core of wave-worn basalt, the core was composed of laval mud and fragments of coral which appeared to have been loose when taken up by the drill, rather than to have been broken off by the cutting tool. This was indicated chiefly by the fact that many of the fragments were blackened over their entire surface and did not show a clean fracture at one end, in the center of which could be recognized the central axis of relatively unchanged coral limestone, such as was characteristic of the coral branches from higher levels up to 50 feet.

The evidence obtained from the drilling at 280 feet from shore showed no especial differences at any level down to the basaltic substratum at 68 feet. The third drilling, 925 feet from shore on the crest of the Lithothamnium ridge, was within 20 feet of the present reef margin. Here one could, from the elevation of the drill derrick, look over the edge of the reef to the channel bottom clothed with a luxuriant growth of *Porites andrewsii, Sclerophytum confertum, S. densum, Nephthya flexile, Millepora,* scattered *Fungias,* etc., and for depths from 20 feet downward to more than 75 feet make an imaginary comparison of conditions as revealed in the material brought up as the core with those to be seen on the bottom of the channel between the reefs on the two sides of the Utelei entrance. Except for the very dense surface layers of the shallow reef and the thinner but apparently comparable layers encountered at several points in the upper 50 feet of the drilling, it was apparent, as the channel bottom was followed down its slope until lost from view in deeper water, that a horizontal line projected from its surface at any level into the solid reef would, in the body of the reef, pass through a mass of limestone almost identical in structural makeup with the skeletal structures of the organisms now living in the channel. After this comparison became more impressive as the drilling operations went on and the channel bottom had been examined more closely by the use of a water glass, a much more thorough study of this botton was made by the use of a diving hood which enabled me to walk over any area desired down to a depth of 30 feet without discomfort, and to depths of 45 feet for a short period of time.

Beyond the vertical, or even under-cut, reef cliff, which was about 15 feet deep and covered over its greater extent with either living *Sclerophytum densum* or spicule rock formed by this organism, on the more gentle slope toward the middle of the channel one walked upon a surface more irregular than that of the shallow reef and lacking any massive organisms except alcyonarian and a few massive *Porites* skeletons. At every step the slender branches of *Porites andrewsii* gave way under one's feet until he stood nearly knee deep among the unbroken colonies on all sides. The soft flexible Nephthyas gently waving in the slow currents, as well as the extreme slenderness of the *Porites* branches, brought out sharply the great difference between ecological conditions at this depth and those on the reef surface at the same time when often heavy waves were racing across the submerged reef with the tide at half flood.

Indeed the one most striking difference between the conditions on the completely alive submerged channel reef (as contrasted with the shallow reef surface now to a great extent devoid of living organisms) and those indicated by the borings is the great amount of sand among the coral skeletons making up the framework of the dead portion of the reef.

On the surface of the reef in the channel along its outer half there is no evidence of the existence of sand until an excavation has been made for at least 18 inches below the surface. Even here many empty spaces occur among the coral skeletons, giving the impression that as yet not enough sand to fill all the interstices had been washed in or formed by disintegration of the skeletons of organisms on the immediate area concerned. Over the inner third of the channel reef, the amount of sand spilling over the reef margin is so great that over considerable areas the stony corals, even *P. andrewsii,* appear unable successfully to cope with this factor. The continuous carpet of living organisms is here lacking in depths above about 30 feet. Scattered *Porites* tufts are separated by areas of sand above which *Sclerophytum confertum* colonies, often of great size, stand up as irregular domes, sometimes several feet in height.

Over the greater part of this channel reef, as indicated by observations at ten rather widely separated points, a small iron pipe could be worked down into the reef for depths in some instances as great as 20 feet, even without the aid of a jet of water flowing through the pipe to wash away the sand.

From this more intensive study of the conditions beyond the present reef margin it seems impossible to avoid raising the question of whether or not, in nearly all discussions of the formation of coral reefs, far too much emphasis has not been placed on conditions now to be observed at the reef margins, where practically all investigators have stated increase in the extent of a reef can alone take place. Barker (1925) emphasizes the growth inward of the surface of a reef as the shore is cut down below sea-level by erosion, due to tides and currents after the reef has grown up to sea-level. His diagrams (*l.c.* p. 1011) and the explanatory statement (p. 1017) indicate that in his opinion a relatively large proportion of the shoreward area of a modern reef consists of a thin veneer of coral overlying a level platform eroded to slightly below low-tide level. The outer portions of a modern barrier reef have grown up in depths less than 30 fathoms on the natural submarine slope. Beyond this depth extension of the reef depends upon the formation of a talus bank upon which the advancing reef is borne.

In support of this idea, he brings forward as evidence his observation that colonies of *Porites frugosa* are in general smaller on the inner portion of a barrier reef than upon the outer portion of the same reef, thus indicating the greater age of the latter. Even if this theory be correct, which does not seem to be indicated by the evidence from the study of the Utelei reef, it throws no light upon the conditions under which the great bulk of a reef develops and, as concerns the growth of corals on the inner portion of a reef, must postulate an environment similar to that existing on the inner borders of the foregoing reefs where, on the reefs about Tutuila at least, practically no growth of corals is now taking place.

Setchell ('26, p. 309) states that no "reef moat" occurs on the Tahitian fringing reefs comparable to that occurring on all the exposed fringing reefs of Tutuila. Consequently under the conditions prevailing on reefs other than those I have studied, the conditions postulated in Barker's theory may be fulfilled.

The idea that the bulk of the material of a coral reef at depths of more than 4 to 6 fathoms (Agassiz, '03) consists of material different from that of the surface layers is not in the least supported by the evidence from the material obtained from our drillings on the Utelei reef. Here the remains of coral-forming organisms brought up from a depth of 120 feet are of the same species as those growing on the reef surface or on the adjacent channel bottom today.

On the basis of the evidence presented by our three borings, this reef for more than half the distance inward from its border rests upon an essentially level platform and is composed of the remains of a collection of organisms apparently of the same species throughout its depth.

Crossland ('28, p. 584) states that, on the Tahitian reefs concerning which Agassiz's ('03) statement was made, "below 5 fathoms corals are more abundant, and may almost cover the bottom, but at 10 or 12 fathoms they die out quite suddenly, and for the next 7 fathoms, *i.e.* as deep as is visible under the best possible conditions, the slope is of barren rock and sand, the sand appearing at this depth for the first time."

Setchell (*l.c.* p. 314) in discussing the contribution of calcareous algæ to reef formation overemphasizes the depth restriction of corals important in reef formation; although for his consideration the actual bathymetric range of these organisms within their observed limits of distribution was of relatively minor importance. His statement (*l.c.* p. 319) concerning the estimation of the time necessary for the formation of a barrier reef that "Any such an attempt to assume a uniform growth rate for either an upgrowing or an outwardly extending reef is subject to extreme doubt *and the upward growth for the last 25 feet is likely to be* *much more rapid, on account of the coral admixture,*" which apparently assumes that below 25 feet the corals play an unimportant part in the process of reef building, is not in the least supported by the evidence presented by the reefs about Tutuila where cores have been obtained.

The marked homogeneity in the structure of the reef below 54 feet, in contrast to the alternating thin hard crusts and very open layers in its upper portion, is the most striking point of difference as revealed by the borings. The presence of the dense layer at the present surface of the reef may be easily explained as the result of the factors introduced when the organisms making up the framework of the reef became subjected to more violent agitation of the water as the reef mass neared the surface. Under this new condition, the fragile skeletons would be broken up and the detritus thus formed would fill the interstices in the framework with relatively large fragments. The corals would become progressively more robust, as can be seen on any reef when specimens of the same species growing in exposed and well-protected situations are compared (cf. Mayer, '24, p. 59; Crossland, '28, p. 723; Wood-Jones, '07, p. 530; *et al.*). In their growth they would tend to spread laterally with much thicker branches rather than to continue to have their most rapid increase in dimensions along the vertical axis, as occurs in the more quiet deeper water. The bringing of the reef surface to a point where, under certain tidal conditions, it was exposed to the sun and air for longer or shorter periods would so change the living conditions that it would be no longer possible for many of the organisms which were the most important elements in the earlier growth of the reef to maintain themselves even at a low degree of activity. Thus a gradual change in the identity of the constituent fauna would be brought about as the less resistant species were crowded out by the more resistant from among their former associates, or as new types, better adapted to the changed environment, became established.

On the reef surface today most of the larger loose fragments of coral debris are overgrown with a veneering of "Lithothamnium"; relatively large areas, at points well in shore from the Lithothamnium ridge bear nearly continuous films of this material, or, as pointed out in the first section of this paper (page 62), are covered completely with a layer of *Sclerophytum* spicule rock to a depth ranging up to as much as 18 inches or approximately one-fourth of the entire thickness of the hard film. As shown by the cores obtained at each of the drilling stations, the surface layer is essentially homogeneous in composition. All the component elements are bound into a dense mass. All of the small cavities are clean-cut and do not show any indication of regularly occupying former interstices among branches of a coral colony, or even those between adjacent colonies, as appears to be true for the deeper portions of the reef revealed by the drillings, and also for the living reef in the channel beyond the border of the Utelei south reef. In other words some mechanical changes, such as the binding together of all the material by Lithothamnium, *Sclerophytum* spicule rock, or the remains of other organisms, have taken place, supplemented perhaps by chemical changes which all together have resulted in the formation of the dense crust over the entire reef surface.

Rarely, over restricted areas, an almost continuous covering of fragile corals is found over the surface of the reef, but this is underlain by the solid limestone which is usually exposed at the surface.

The origin of the dense layers at varying levels below the surface crust is less easily accounted for. It would seem necessary to postulate the intervention of some ecological factors which, over an extended period of time, would have interfered with the normally rapid upgrowth of the reef-forming organisms. Under such conditions a more solid layer would result from the accumulation of material, whether coral debris or skeletons of other forms, at a rate proportionately much greater in comparison to the rate of upgrowth of the reef than would take place under conditions more favorable for the rapid growth of the organisms which have evidently constituted the framework of the dead reef as they do the upper surface of the channel reefs today.

The occurrence of such dense layers at depths of 14 feet, 19 feet, and 46 feet, with two less resistant but clearly marked lines of solidification between the two last-mentioned depths, would necessitate the recurrence of the conditions postulated above with intervening periods when conditions favorable for more rapid growth of corals were maintained.

There is no evidence available from which the relative duration of the period necessary for the formation of one foot of the more dense reef material as contrasted with 8 feet of the loose reef which exists between the two upper hard crusts can be estimated. Any attempts to check back in time for a definite depth in the reef, or indeed to estimate even roughly the time necessary for the formation of a modern reef made upon the basis of the rate of growth of coral forming organisms on the reef surface today must, in the light of the existence of these dense and loose layers, be subject to grave suspicion.

The suggestion that the presence of these several dense layers might be caused by variations in sea-level from time to time during the history of the development of modern reefs, when present-day surface conditions would have been approached at these varying levels, is enticing when considering a region where there are many evidences of alternating subsidence and emergence.

The interpretations of Chamberlin ('24), Mayor, ('20), Daly, ('20), *et al.*, place the time of the interpretable fluctuations of sea-level before rather than during the time of the formation of the modern reefs, and the most recent movement is stated to be a lowering of sea-level which occurred before the formation of the present fringing reefs (Chamberlin, *l.c.*, p. 168).

Since a change of sea-level, such that the young reefs would be brought to the surface, would result in the formation of a dense crust, the thicknesses of these layers would to some extent be a measure of the relative duration of the period during which these conditions had been maintained.

Whatever may have been the environmental factors responsible for the formation of such dense layers, a set of conditions operative over varying lengths of time has recurred at intervals during the formation of the upper portion of the reef mass. The effects of the changed environment would be much more clearly distinguishable in the structure of a reef, such as the Utelei reef where, as noted by Mayor (*l.c.*, p. 13), the chief component madrepore *Porites andrewsii* is much more fragile and slender than the *Acropora* growing on a wave-beaten reef. The position of Pago Pago harbor is such that the incoming ocean water sets along the East reef, while an outgoing current flows along the West side of the harbor. This condition has been the most important factor in determining the faunal constitution of the reefs on the two sides of the harbor. This faunal makeup has given to the Utelei reef the structural characteristics already noted, while the Aua reef, through which a boring was made under the direction of Dr. Mayor, was found to be composed of more dense and consequently much more homogeneous material throughout its depth. Relatively little sand, as compared to conditions on the Utelei reef, was encountered, although enough was present in the upper 50 feet of the reef to make advisable the use of a casing to this depth.

Under such conditions there is little possibility of recognizing by any structural characteristics of the reef limestone any differences in the rate of accumulation of the reef-forming materials so strikingly shown in the progress of the boring through the generally loose material of the Utelei reef. That such alternating periods of greater and lesser rapidity of upgrowth have occurred on reefs where, because of their composition, no such striking evidences are found to exist as occur in the more protected reefs, is, it would seem to the writer, assured since the determinative factors, whatever their nature, could not be readily conceived as being important enough to have affected the entire reef area on the protected side of the harbor and yet not be operative throughout a much wider area.

A study of the structure of the channel reef at depths comparable to the levels occupied by the dense layers of the exposed reef would be very enlightening as indicating the influence of the factors responsible for the formation of the hard crusts, but no such data are available at present.

The chemical constitution of the reef-forming material as shown by the study of the cores obtained from the borings at Funafuti (Judd, 1904) indicated that in the upper 150 feet of this reef, *i.e.*, the modern portion, the proportion of magnesium carbonate was greatest at depths from 10 to 40 feet. Below the last-mentioned level minor fluctuations only were found, until at 638 feet a sudden rise from 2.44 to 20.44 per cent occurred.

In the material from the Utelei reef, many fluctuations in the $MgCO_3$ content occurred in the upper levels of the reef because of the marked difference in chemical constitution of the materials to be encountered. Spicule rock formed by *Sclerophytum confertum* contains normally a concentration of $MgCO_3$ as high as 36 per cent, according to the analyses made by F. W. Clarke on material from Samoa, but so far as I am aware unpublished. *Sclerophytum densum* has a $MgCO_3$ content of about 10 per cent. The Samoan Lithothamniums vary from 15.25 to 16.46 per cent according to Lipman and Shelley (1924). The Madreporarian skeletons in marked contrast to the foregoing groups show a uniformly low concentration of $MgCO_3$. Clarke and Wheeler (1922, page 8) list the analyses of 30 species of madrepores, mostly from the Caribbean region, of which only one had a $MgCO_3$ content above 1.0 per cent.

With such exceedingly variable elements entering into the composition of the limestone, in at least the upper portion of the reef, information obtained from chemical analyses of material from this level would be indicative of little more than the identity of the organism, the skeletal remains of which it represented. Below the level of the upper dense layer a much more homogeneous chemical constitution was found throughout the more open, sand-filled layers. The material from the thinner dense layers at 14, 19 and 49 feet was again more variable in chemical characteristics.

Throughout the remainder of the reef, down to the substratum, the chemical composition varied very slightly, so that there was not recognizable any chemical change at all comparable to the striking physical changes observed in the material from the lower levels of the reef. While the material between the coral branches had taken on the appearance of a dark-brown coarse mud instead of being clean cream-colored sand as in the upper part of the reef, and even the greater part of the coral skeletons, or *Sclerophytum* spicule masses, had undergone a similar change in color, chemical analysis showed that there had been no consistent alteration in the chemical constitution of the "limestone," in spite of the altered appearance. Although such striking physical changes had been undergone, it was still possible to recognize the identity of nearly all the skeletal remains, as madrepores, alcyonarian spicules, Lithothamnia, etc. Wherever a sample was clearly of uniform composition, its chemical constitution as revealed by analysis differed only slightly from that of the most recent skeletons of the same species taken from the surface of the reef.

PART III

BEARING OF EVIDENCE OBTAINED BY BORING THROUGH UTELEI REEFS UPON THEORIES OF CORAL-REEF FORMATION

The literature dealing with the origin of coral reefs has become enormous and has in relatively recent years been reviewed in detail by a number of writers on this subject as Davis ('14), Daly ('15), Mayor ('18), Vaughan ('19). As a result of these studies three distinct theories have emerged, with clear-cut definiteness, which may be characterized as: (1) the Theory of Subsidence, first put forward by Charles Darwin ('42) to explain the formation of atolls, and more recently elaborated by W. M. Davis in a long series of papers; (2) The Theory of Solution most closely associated with the name of Sir John Murray (1879-80), but strongly supported by Alexander Agassiz ('03); and (3) Theories of Planation, which postulate the existence of an antecedent wave-cut platform upon which coral reefs have been established. This last-mentioned theory has been supported by Gardner ('02), Andrews ('02), Vaughan (*l.c.*) *et al.*

The Glacial Control theory of Daly (1915), which as the name indicates more specifically refers to the formation of modern reefs in post-glacial time, is hardly more than a special instance under the general classification of theories of planation. It nevertheless offers an opportunity for a more definite checking by actual determination of the relation of modern reefs to the substratum than do the more broadly stated postulates of the theory of planation as a whole, and upon its validity definite information is given by the results obtained from my borings through the Utelei Reef.

Chamberlin (*l.c.*) has pointed out a number of weaknesses in the glacial control theory and called attention to the fact that Daly in a later ('19) paper had receded from his original position, in which probably more planation during Pleistocene time was demanded than could reasonably be accounted for.

Since the real crux of the problem is to be found in the nature of the substratum upon which the reefs rest, it is of the greatest importance to ascertain whether the underlying volcanic rock shows the natural island slope or whether the reef rests upon an essentially flat wave-cut floor.

The results from our three borings along a line across the Utelei reef give a definite answer to this question, as applied to a reef situated well within a harbor. Here, because of the configuration of the land, marine erosion would be less pronounced than upon the more exposed shore line of the island. At a distance of 280 feet from shore the substratum was encountered at a depth of 68 feet, which would seem to indicate that at this point the reef rested upon a natural slope. At this distance from shore along line No. 1, protection against the most energetic wave action would be afforded by Blunts Point which extends out beyond the Utelei reef.

The fact that at both the borings at 575 feet from shore and at 925 feet the basalt substratum was reached at a depth of essentially 20 fathoms shows clearly that, for the distance of at least 370 feet in from the present reef margin, planation to an essentially level platform had been accomplished before the growth of the modern reef had begun. How far shoreward beyond the location of the intermediate bore hole the planation had progressed is, of course, purely a matter of conjecture. Definite information upon this point would be of interest, but the borings were undertaken primarily to secure data concerning the structure of the reef, and after these three had been completed no time was available to make the supplemental bores made desirable by the nature of the information already obtained.

Whether or not the shoreward portion of the reef consists of a thin veneer of coral as postulated by Barker could have been easily demonstrated also had time been available. The greater depth at which the substratum was reached in the boring on the Aua reef might be interpreted as indicating that the planation at the depth indicated on the Utelei side of the harbor was not of general occurrence. On the other hand, the character of the topography of this immediate region appears to justify the conclusion that the natural slope of the east side of the harbor would carry the laval substratum to the depth actually found at the distance from the shore at which the boring was made.

While an available location for drilling on the exposed seaward reefs about an island is rarely found, an opportunity to accomplish this without any of the inconveniences usually to be encountered was provided on the south shore of Tutuila. Here a relatively recent laval flow extends completely across the reef and nearly to the edge of the deeper submarine platform which surrounds the island. A series of borings through the lava and the limestone of the dead reef below until the basal lava was reached would give definite information concerning the character of the surface of the substratum upon which the reef had been built up and would thus present data from which could be derived a final answer to the question of the validity of a theory of planation, if not specifically to the glacial-control theory, for the formation of modern reefs.

The unique opportunity here offered for testing the validity of what has come to be probably the most widely accepted theory of coral-reef formation makes it especially to be regretted that this particular work already projected by the late Dr. Mayor should have been, up to the present time, impossible of accomplishment.

If, as seems apparent from the evidence advanced in this section of the present paper, modern coral reefs have usually been built up upon a fairly level submarine platform, the idea that a modern barrier reef has grown out from the shore on a sloping substratum and, for a considerable portion of its extent must have awaited the formation of a talus bank formed from material broken from its own edge before it could advance seaward, is not tenable. On the contrary the upward growth, at least until a point near the surface was reached, would proceed at a fairly uniform rate over the entire reef area. The volume of water passing over the reef surface under such conditions would be more than sufficient to assure adequate aëration and the rapidity of the change of water (*i. e.*, the currents) sufficient to assure a fairly even distribution of the food supply. Only when the reef had come quite near to the surface would the distribution of oxygen and food or the relative force of wave action appear as environmental factors sufficiently important to affect the rate of growth on different parts of the reef to a marked degree.

The similarity in reef structure shown by the borings through the Utelei reef, the inner and the outermost of which were 645 feet apart, testifies strongly to the fact that throughout this area conditions must have been uniform during the growth of the reef. The presence of the successive dense layers at the same depth at each of the borings is especially strong evidence that the entire surface of this reef was at the same level when acted upon by the factors, whatever they may have been, which caused the formation of these layers which differ so markedly in character from those immediately below or above them.

Where a very broad platform was involved, as in the formation of large atolls, it is quite conceivable that conditions near its margin would be more favorable for coral growth to an extent sufficient to limit the area of rapid upgrowth of the reef to an area relatively narrow, as compared to the extent of the platform, but actually wider than the extent of any modern barrier reefs.

Neither on the reefs about Pago Pago harbor, nor so far as observed on any reefs about Tutuila, was there evidence of the retrogressive changes in the reef margin such as Crossland ('28) has stressed so strongly for the reefs of Tahiti. Here again emphasis is placed upon conditions operative only after the great mass of the reef has been formed and a set of environmental conditions not operative during the earlier stages of its growth has been introduced.

In relation to the questions raised by Crossland ('02, '28a, '28b), as to whether or not coral growth has for modern species passed its maximum, as well as in relation to the formation of the reefs in exposed locations, there must be considered the point concerning how fairly the conditions found to exist on the well-protected Utelei reef are representative of those occurring in other locations.

While, as indicated by the cores obtained from the Aua reef, the more exposed reefs are made up of much more dense limestone in which less fragmentary material is found, and consequently the response to variations in environment are less easily followed, it seems reasonable to suppose that the more protected reefs may be looked upon as simply more loosely knit because of the absence of the response to severe wave action, and consequently to represent a more easily interpretable but none the less representative result of the activities of reef-forming organisms.

When more severe wave action must be withstood, the relative importance of the Lithothamniums may be much greater than on the sheltered reefs, but that under any conditions they ever reach the importance ascribed to them by Finckh ('04, p. 147) and by Setchell (*l.c.*, p. 319) is doubtful. At best the conditions under which they would be of the greatest importance would be reached only when, after the formation of the great bulk of a modern reef, its surface had come near to sea-level.

GENERAL DISCUSSION

IMPORTANCE OF ALCYONARIA IN FORMATION OF PACIFIC REEFS

A comparison of the part played as reef builders by Alcyonaria in the reefs of the Atlantic and Pacific oceans brings out immediately a striking contrast. On the Atlantic reefs nearly all the species set free on the death of the colony small spicules which as distinct individuals are added to the "coral sand." While in many localities the amount of material thus contributed to reef formation is large (Cary, '18, p. 352), it does not at once become an integral part of the reef limestone, but must, along with other fragmentary skeletal remains of marine invertebrates, be later consolidated into the general limestone mass.

On the Pacific reefs, however, Alcyonarian spicules as separate particles are less often found. Vaughan ('18, p. 224) states "*Alcyonaria*—This group is given a caption as it is important in the Bahamas and Florida. Spicules occur in nearly every, if not every shallow water sample I have examined from these areas, but there are few or none in the Murray Island specimens. The abundance of such spicules in samples from the former area and their scarcity or absence in samples from the latter constitute the most striking difference between reef samples from the two areas. However, in other Australian reef areas they are probably important, as

Alcyonaria are abundant in many places, and especially in areas where silt is being deposited, for there *Sarcophytum* and *Xenia* grow plentifully."

The absence of spicules in bottom samples of loose material from the Samoan reefs would not necessarily indicate the absence of Alcyonaria, but rather that the most common Alcyonaria—species of the genus *Sclerophytum*—have their spicules cemented into a solid mass more dense and stronger than the skeleton of any madrepores except a massive *Porites* or *Siphastrœa.* This spicule rock is also more resistant to disintegrative forces than the madrepore skeletons which it often covers over and protects on the reefs.

On the Samoan reefs species of *Sarcophytum* and *Nephthya*, which contain a small bulk of uncemented spicules (see table 2), occur in well-protected areas only, and their spicules would consequently not be a general constituent of bottom samples taken at random on the reefs.

On the more exposed reefs in Pago Pago harbor and about the periphery of the island, *Sclerophytum densum* is a regularly occurring component of the fauna on the vertical surface beyond the rim of the reef. Here its skeletal remains, as a veneering of spicule rock, form a most important binding material to support or consolidate the skeletons of the madrepores which it has overgrown, and in many instances killed. Although abundant on the vertical surface of the reefs, few colonies occur on the horizontal surface, apparently finding the conditions where they would be subject to the full violence of the surf uncongenial.

In more protected areas they may become the dominant element of the reef and constitute by far the greater bulk of its framework, crowding out, by their more rapid growth in area, even the madrepores adapted to the less favorable conditions.

Although on the several coral pinnacles rising from deep water in Pago Pago harbor the outer surfaces are coated with *S. densum* to a depth as far as the eye can reach, it would seem that on the reefs where now the Alcyonaria, especially *S. confertum*, are the dominant forms they had become progressively more important as the reef mass had grown closer to the surface. This is especially well illustrated in the channel between the two Utelei reefs, where as one goes down from the reef margin, immediately below which an almost continuous carpet of *Sclerophytum* is found, the proportion of the area covered by Alcyonaria becomes less and less until finally only a few scattered specimens occur. However, in the cores from the borings on the Utelei reef, samples of spicule rock were brought up from near the bottom o the reef, so that the occurrence of *S. confertum* at the foot of this modern reef, at least as occasional colonies, is assured.

The Gorgonaceæ, so common on the Atlantic reefs, were not found about Tutuila in any of our collecting. At Tau island many examples of the bare central axis of some of these forms were washed up on the beaches, coming apparently from deeper water. A "scrub" of gorgonians was described as occurring on the outer slopes of Funafuti (David, Halligan, and Finckh, '04, p. 154) in depths from 140 to 200 feet, so the occurrence of these forms may not be uncommon. That the abundance of *Sclerophytum* observed about Tutuila is not an unusual occurrence is indicated by the observations of Gardiner from the Maldives, Pratt (*l.c.*), and th observations of many other collectors at various localities in the Pacific region. That their importance as reef builders has been overlooked is apparently due to the fact that the character and extent of the limestone composed of their closely cemented spicules had not been fully appreciated, as can readily be understood when the surface layers of a colony is composed of relatively soft, smooth tissues.

SUMMARY

(1) The character of the physical environment is described for Pago Pago harbor in general and for the reefs at Utelei in detail (pages 58-60).

(2) The relation of the character of the reef surface to the distribution of the Alcyonaria is considered in order to account for the observed occurrence of these organisms (page 60).

(3) The identity and structural characteristics of the four important species of Alcyonaria found about the island of Tutuila, and also their relative spicule content, are discussed (pages 60-62).

(4) The distribution of the several species of Alcyonaria occurring on the Samoan reefs is directly determined by their capacity to adjust themselves to environmental conditions at different locations in the reef area. *Sclerophytum confertum* is the most resistant form and consequently the most widely distributed. It occurs in both the most favorable and most unfavorable habitats. *Sclerophytum densum,* the least resistant species, is confined to well-aërated water. *Nephthya flexile* occurs in quiet water only and can withstand silting better than the other species. The capacity of resistance to high temperature—asphyxiation—of the four species conforms to their general resistance to adverse conditions; while their rates of respiration show no correlation with resistance (pages 63-66).

(5) The relative areas of reef surface covered by living Alcyonaria were determined by laying out lines of 25-foot squares across several reefs. The results of these determinations are shown in tables, charts and graphs (pages 66-71).

(6) The growth rate of the four species studied was determined under natural conditions by measuring specimens *in situ* on the reef and repeating the measurements from time to time. Since the increase in area is the most important record, both in relation to other organisms and to the amount of reef surface covered by limestone composed of spicules of these forms, this record was alone kept at the time of the later measurements. At the end of approximately 15 months the areas had increased as 1:2.35 for *S. densum;* 1:13.49 for *S. confertum;* 1:3.96 for *Sarcophytum latum* and 1:6.56 for *Nephthya flexile.* The maximum increase was as 1:102.0 (*S. confertum*) (pages 72-79).

(7) As one phase of the study of the composition of the reef, a determination of the identity of the skeletal remains over a square yard of surface was made at 10-foot intervals along a line across the Utelei reef. Here at 83 of the 94 stations Alcyonarian spicule rock was found to occur. Similar observations on other parts of this reef, as well as on several other reefs, confirmed the impression that this material was of common occurrence (pages 80-82).

(8) Drilling through the reef, both from the mechanical conditions encountered and the evidence obtained from the material brought up as a core, showed that this reef has a dense surface layer some 6 feet in thickness followed by about 8 feet of open coral containing much sand. Another dense layer occurs at 14 to 15 feet. From this point to a depth of 46 feet, loose material with freely flowing sand was encountered, then a very hard layer 2 feet in thickness. From approximately 50 feet, down to the substratum, the reef was made up of a relatively open framework with the interstitial material transformed from sand to a dark-brown, rather coarse, sticky "mud." The coral skeletons have also undergone considerable change in color, so that only a narrow central core of white limestone remained. The structural identity of the different species was, however, retained in both madrepores and alcyonarians.

At the first bore hole, 585 feet from shore, the base of the reef, where a core of wave-worn basalt was obtained, was reached at a depth of 124 feet. A specimen of *Siphastrea* from 121 feet, which had apparently been buried in laval mud, appeared as fresh as a similar section from the surface of the reef (pages 82-85).

(9) The conditions observed on an actively growing reef in a channel beyond the boring at 925 feet from shore offers an opportunity for a comparison with those presumably in force when the now exposed reef was being formed. In the light of this evidence, the conclusions of several writers on the subject of coral-reef formation are discussed and a tentative explanation of the formation of the alternating dense and open layers is offered (pages 85-89).

(10) The various component elements of the reef limestone differ markedly in their $MgCO_3$ content. Very little except the origin of any sample of material, from whatever depth obtained, could be determined by chemical analysis (pages 89-91).

(11) The evidence obtained by making a series of three borings on a line across the Utelei reef gives certain proof that the seaward portion of this reef rests upon a wave-cut platform, roughly 20 fathoms below present sea-level. The topography of the harbor at this point is such that marine erosion would be retarded on this shore, so it is probable that on the more exposed shores planation would have been more complete. *The desirability of further borings at a point made especially available by a recent laval flow is pointed out* (pages 92-93).

(12) If modern coral reefs have been built up on wave-cut platforms, their upgrowth would take place at a relatively even rate over their entire surface. This conclusion is supported by the occurrence of the various dense strata at approximately the same depth at the three points where borings were made on the Utelei reef (pages 93-94).

(13) The characteristics of the limestone contributed to reef formation by the Alcyonaria on the Pacific as contrasted to Atlantic reefs differ greatly in that *Sclerophytum* spicules form a dense homogeneous mass, which is left as a unit on the death of the colony. Indeed at the base of an old colony of *S. confertum*, a mass of spicule rock several feet high may be formed. The Gorgonaceæ, characteristic of the Atlantic, leave only free spicules when the colony disintegrates, and these elements, so characteristic in appearance, are striking components of bottom samples but far less important as a contribution to limestone formation than the spicule rock of *Sclerophytum*. The wide distribution of the Alcyonaria on the Pacific reefs, when considered in relation to the character of their skeletal remains, assures to them an important place as reef formers (pages 94-95).

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Plate 1

Fig. 1—View across the Utelei South Reef with camera set up at point of origin of lines 1 and 5. The drill platform, in middle distance, is set up for middle boring, 675 feet from shore, on line 1. Goat Island appears as a low-lying dark mass immediately to right of drill. The edge of the native village of Utelei appears among palm trees at extreme left.

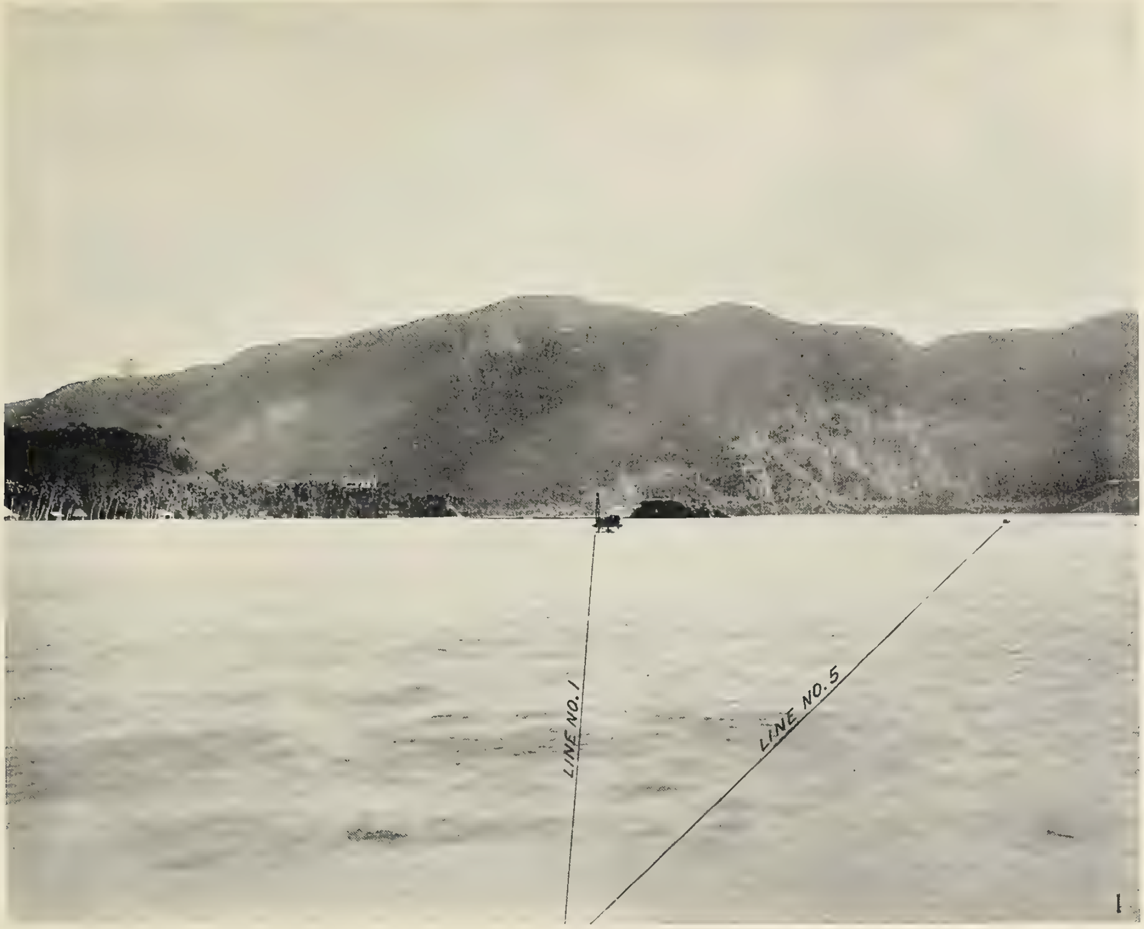
Origin of line 2 is at right hand (east) end of culvert near middle of roadway connecting Goat Island with the larger land mass. Brotherhood School which marks the bearing of line 4 is the white building directly over drill platform. Sharp notch in the rim of hills near right of the background is the pass to Vatia on the north shore of the island.

The location of line 1 and line 5 are marked in black across the reef.

Fig. 2—View to southward, across Utelei reefs with camera set up on the West end of upper platform of Goat Island. Part of the village of Utelei appears in right half of the picture. Blunts Point forms the left hand (Eastern) end of the land shown.

The deep water of the channel between the North and South Reefs occupies the middle distance, while the outline of the entire South Reef is marked by a rim of "white water."

The drill platform, at the 675 foot point on line 1 is recognizable in the distance.


LINE NO. 1
LINE NO. 5
1

2

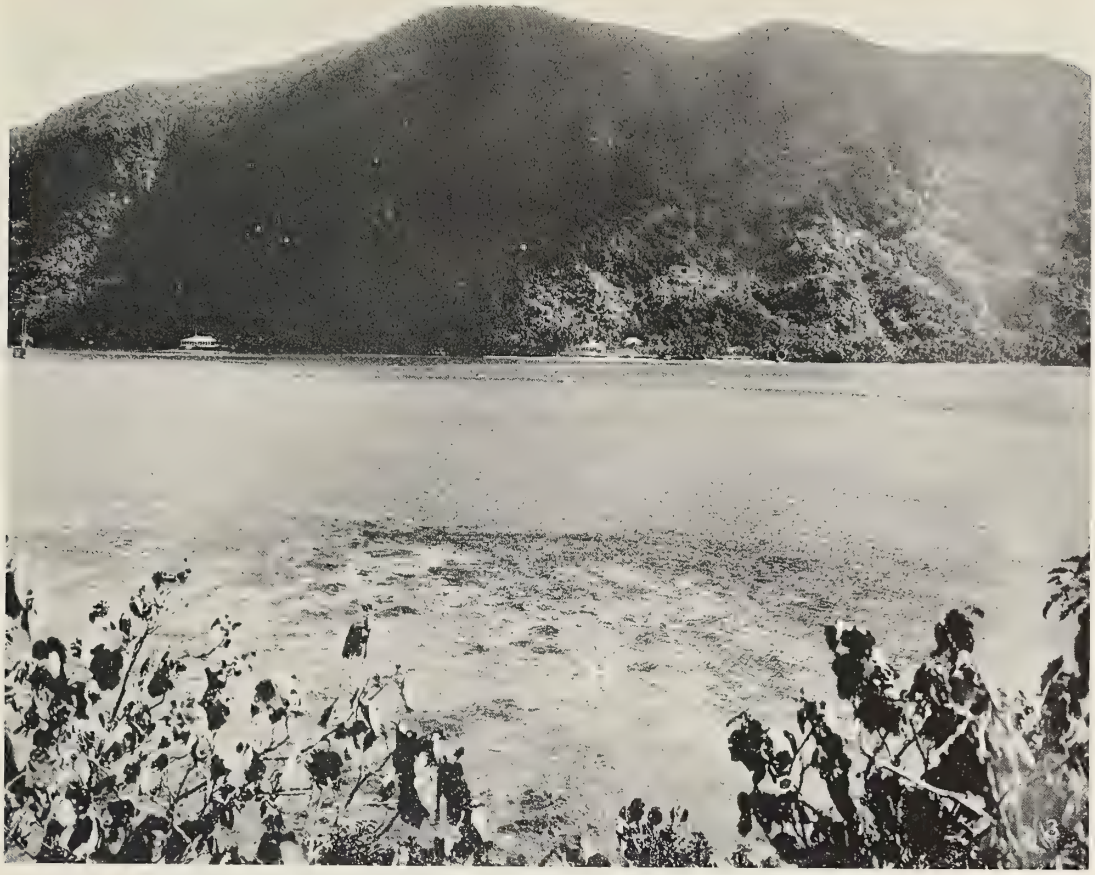


FIG. 3—View across reef from origin of line 2. Dark areas on outer portion of reef are occupied by living *Sclerophytum confertum*.

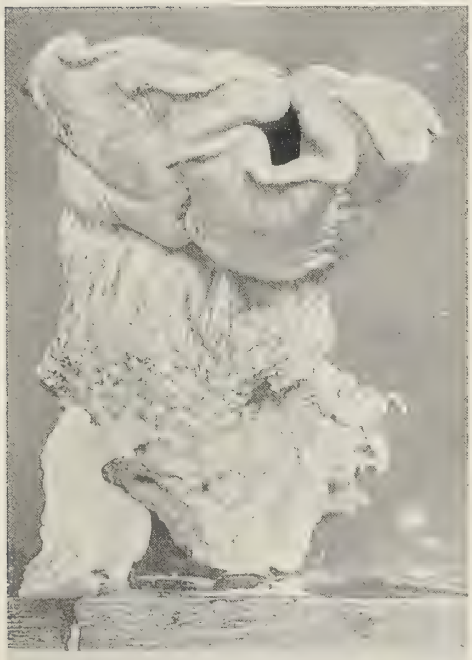


FIG. 4—A specimen of *Sarcophytum latum* in side view. natural size.

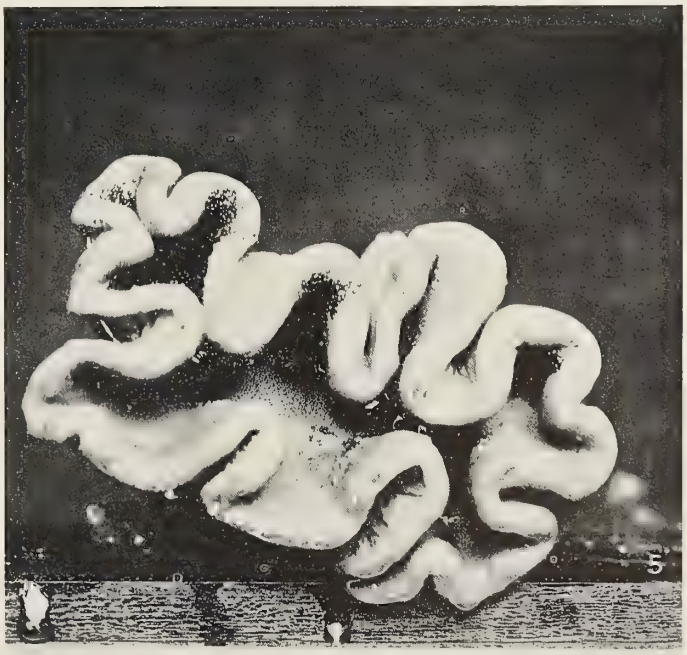
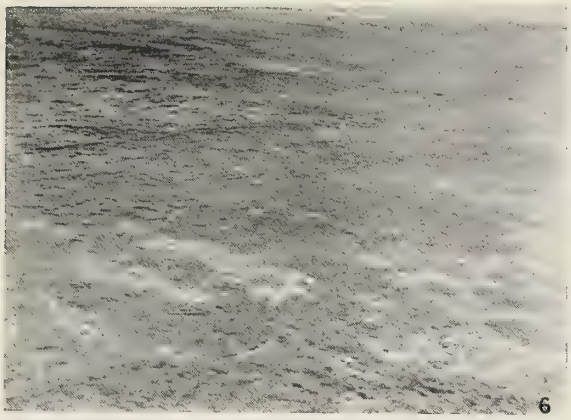


FIG. 5—The same specimen viewed from above, natural size.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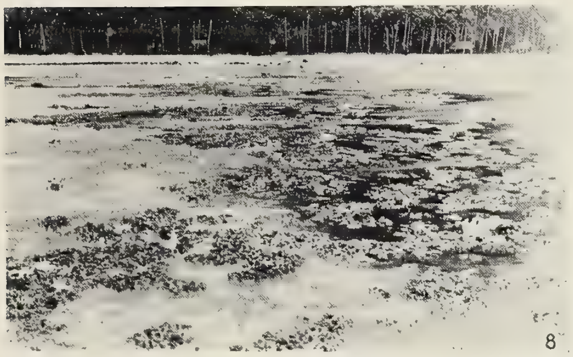
FIGS. 6 and 7—Views from drill platform when set up for second boring at the 675-foot mark on line 1, looking westward to show proportion of reef surface covered by living Alcyonaria.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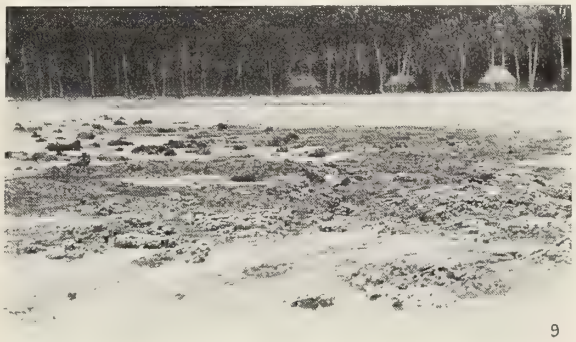
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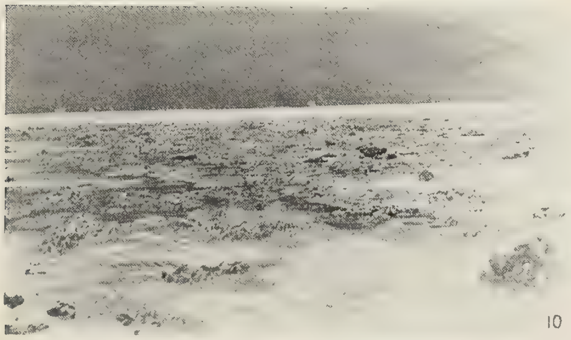
FIGS. 8 and 9—Views across inshore border of Utelei South Reef at low tide to illustrate the growth habit of *Sclerophytum confertum.* The village of Utelei can be seen in background.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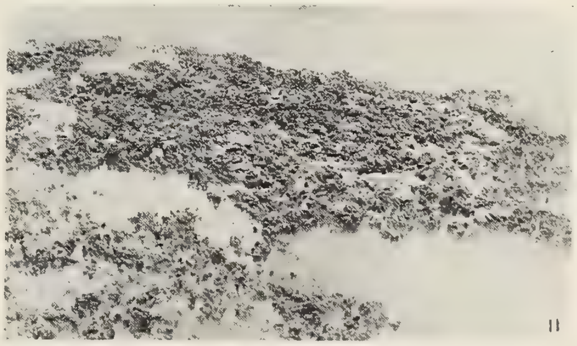
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FIGS. 10 and 11—Two views from drill platform, when set up at 925-foot mark on line 1, across reef to show character of Alcyonarian fauna.

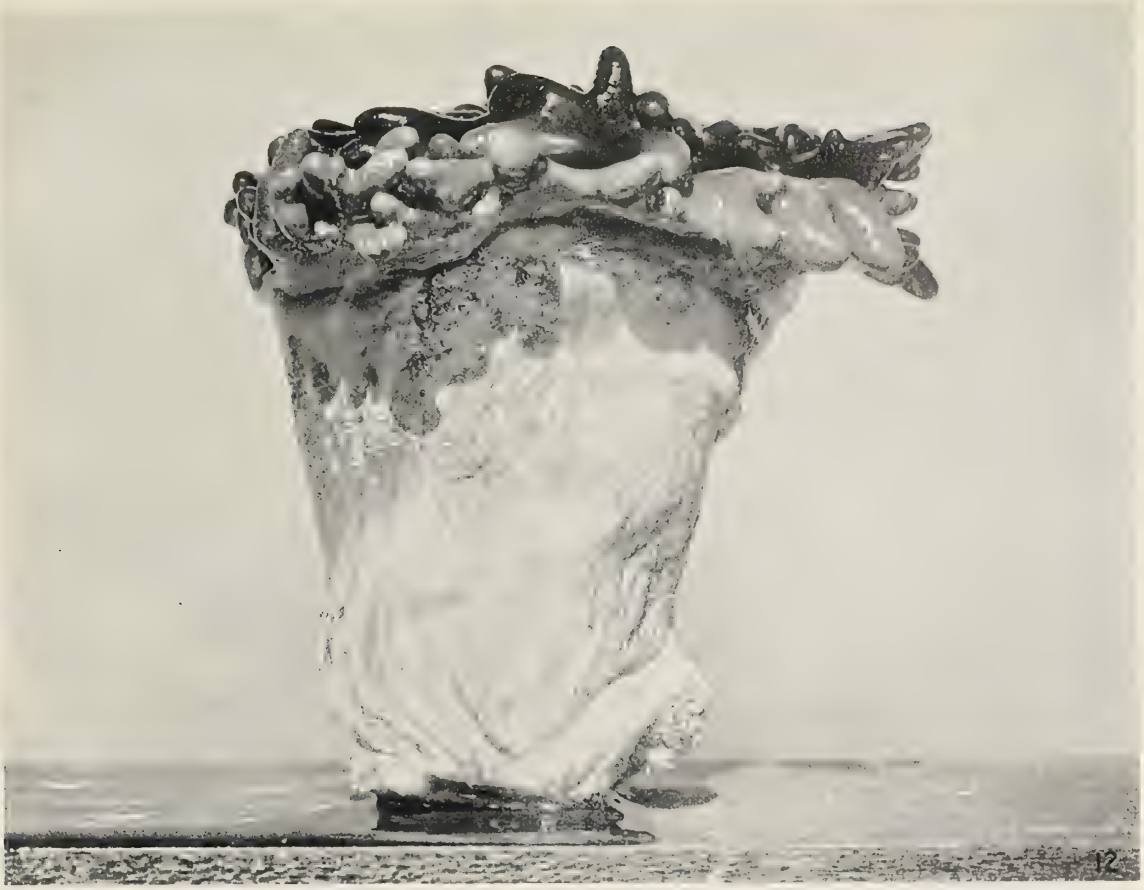


FIG. 12—Side view of a specimen of *Sclerophytum densum* (Whetlegge) natural size.

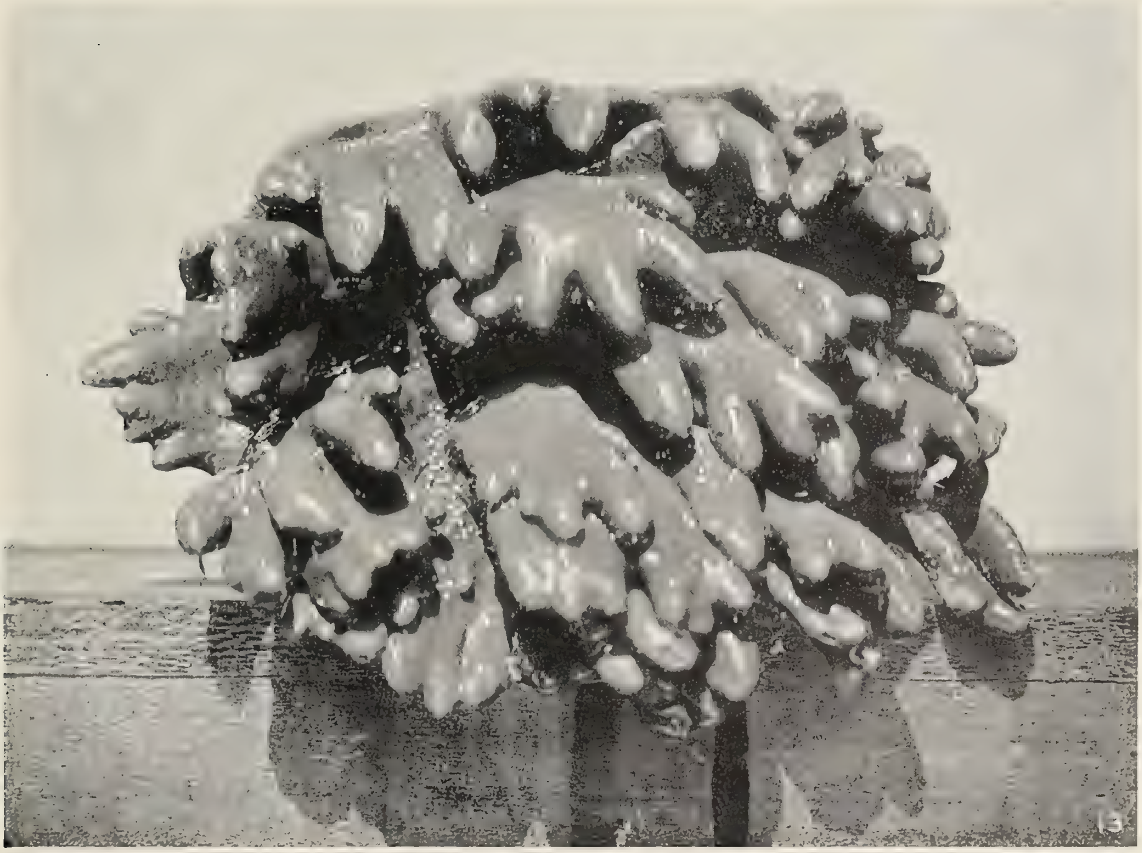


FIG. 13—Top view of a slightly larger specimen, natural size.

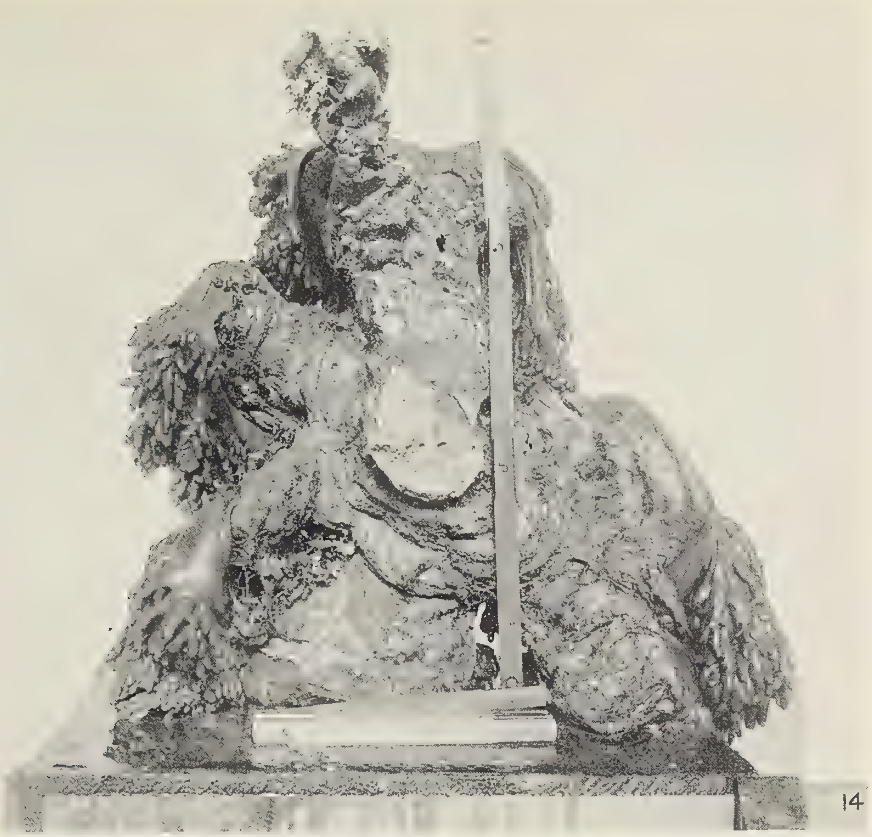


FIG. 14—View from below of a specimen of *Sclerophytum confertum* (Dana). This growth habit, with a large mass of living tissue and heavy underlying spicule mass attached by a slender "stalk" to the substratum occurs regularly when this species is found in shallow water, about one-third natural size.

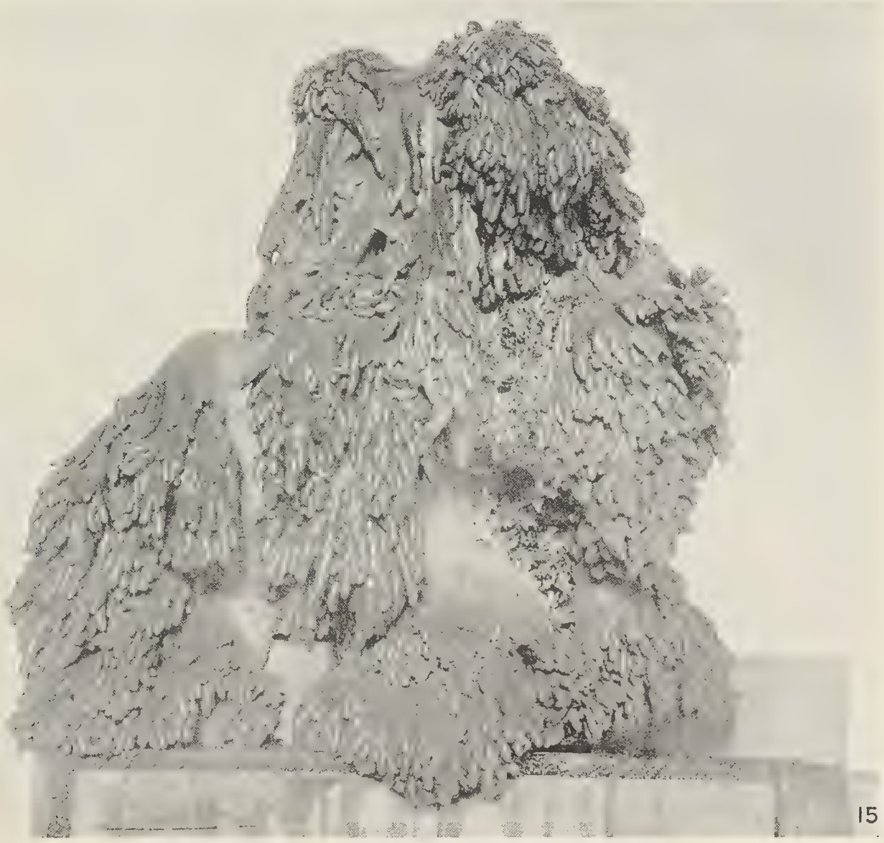
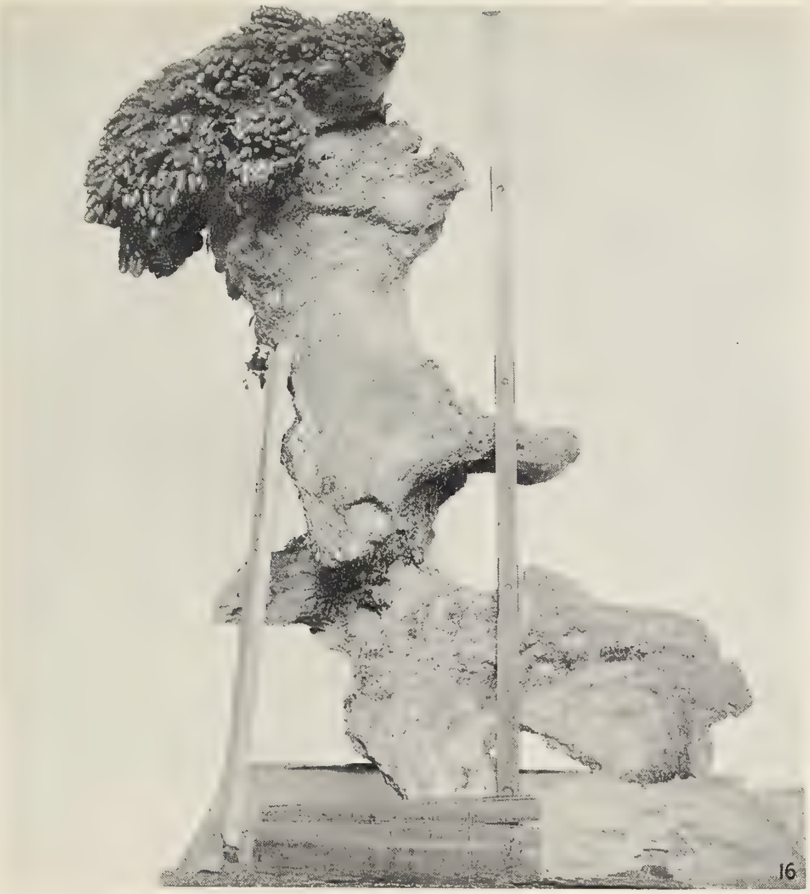


FIG. 15—Top view of the specimen shown in figure 14.



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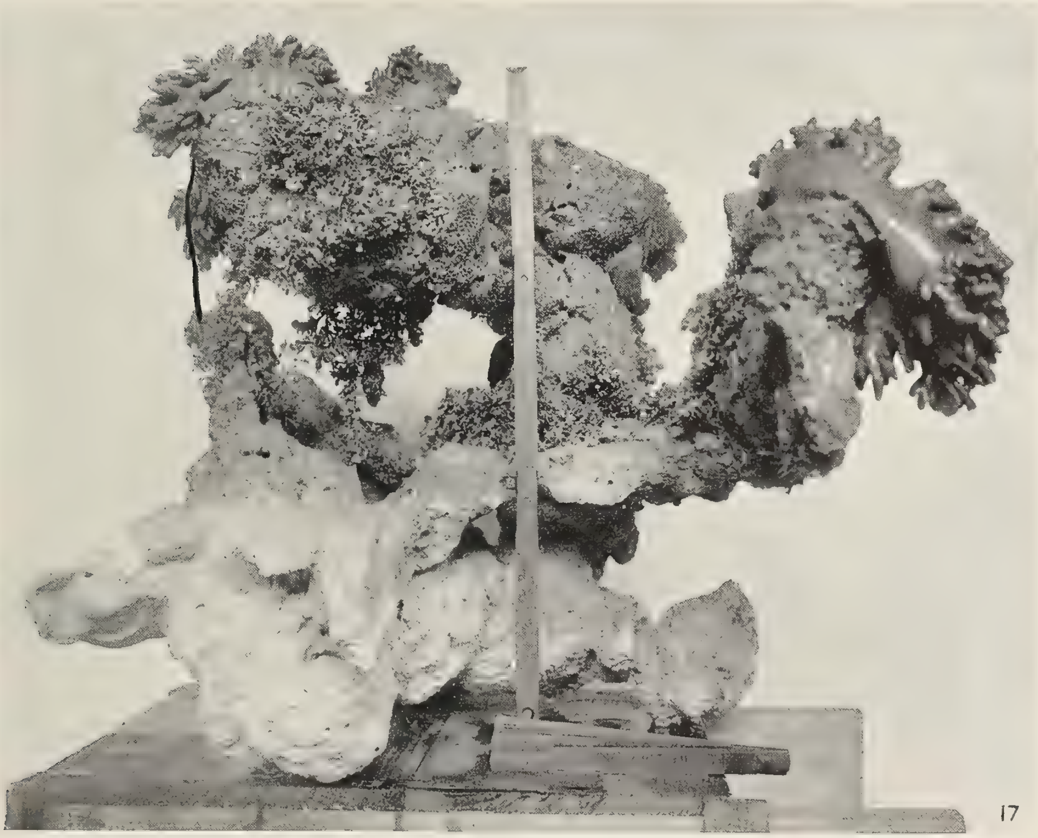

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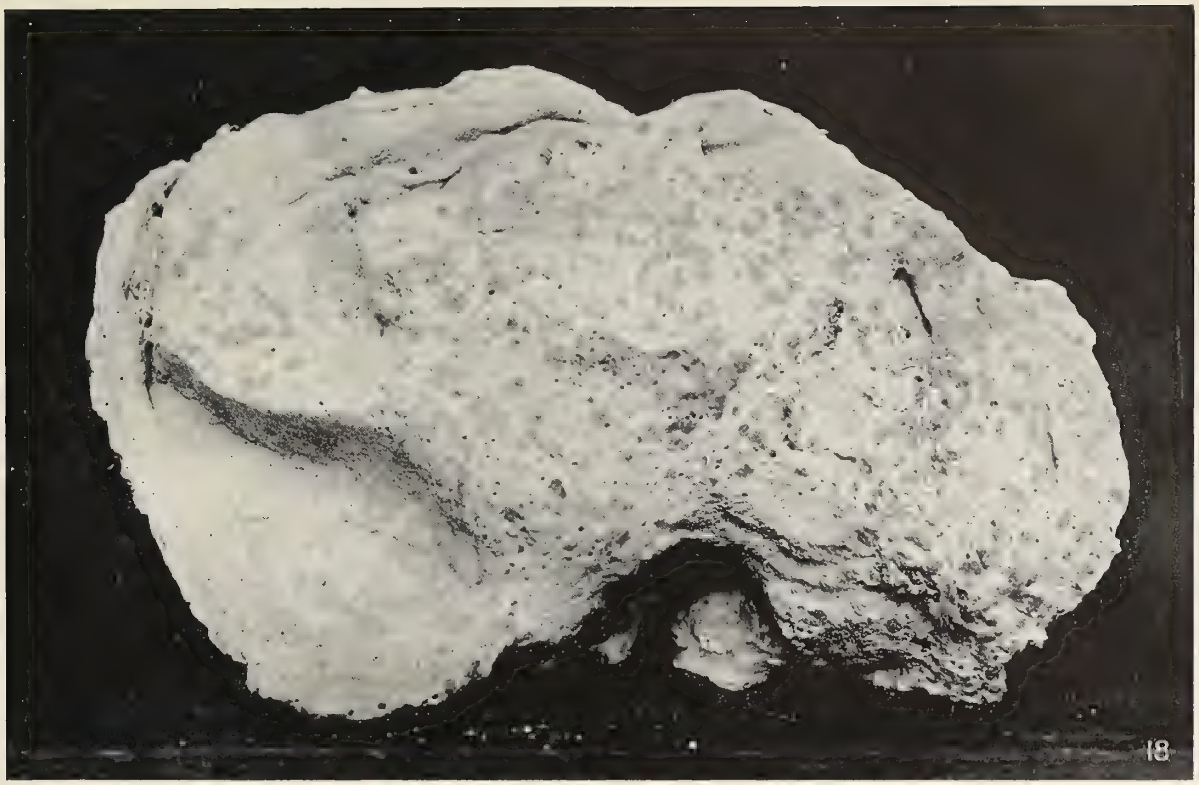
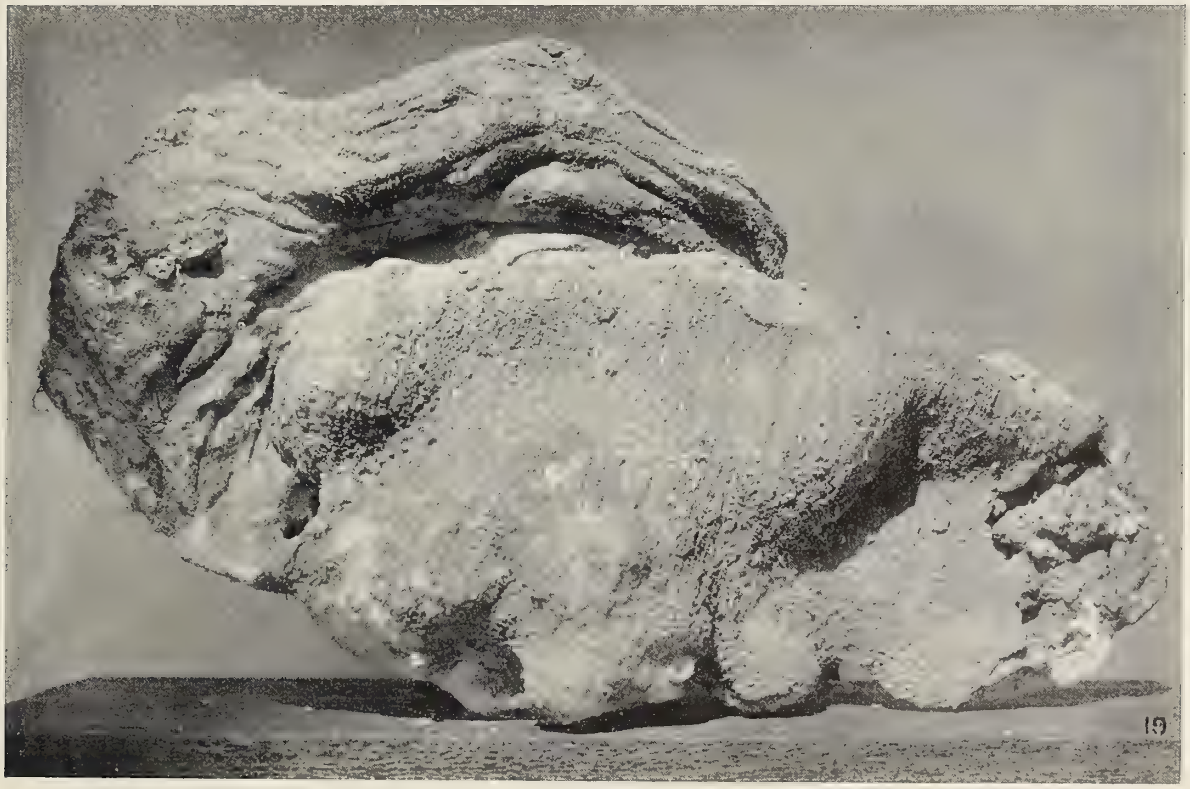
FIGS. 16 and 17—Side views of two specimens of *Sclerophytum confertum* to illustrate its habit of growth in deeper water or where there occurs an abundance of shifting sand. About one-third natural size.





Figs. 18 and 19—Two specimens of spicule rock formed by *Sclerophytum confertum*. These almost scale-like structures are found commonly on shallow reefs and undoubtedly result from breaking from their attachment of specimens such as shown in figures 14 and 15, with subsequent smoothing off of sharp angle at point of separation.

IV

FORMED COMPONENTS AND FERTILIZATION IN EGG OF THE SEA-URCHIN LYTECHINUS VARIEGATUS

ESTHER C. HENDEE

With two plates

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FORMED COMPONENTS AND FERTILIZATION IN EGG OF THE SEA-URCHIN LYTECHINUS VARIEGATUS

INTRODUCTION

This investigation of the eggs of *Lytechinus variegatus* was undertaken in an attempt to determine whether or not any substance in the egg, preserved by the Champy-Kull method, is concerned in the process of fertilization. The experimental work of Chambers ('21) on the starfish egg and of Just ('23) on the egg of *Echinarachnius* has made clear that the substance, or substances, essential for fertilization are localized in the cortex of the egg. A study of *Lytechinus* eggs, preserved in strong Flemming's solution, was being made by another student in the laboratory at the time the present investigation was in progress; the results indicated that in such eggs there is a cortical substance, stainable in iron hematoxylin and having the appearance of a precipitation product, which is present in mature, unfertilized eggs, but which disappears upon fertilization or parthenogenetic activation. The present study, involving a comparison of fertilized and unfertilized eggs fixed by the method of Champy-Kull (Lee, 1924, p. 321), indicates that a substance concerned in the process of fertilization is preserved by this technique as well.

The investigation was suggested by Professor David Hilt Tennent, and it is a pleasure to express my gratitude to him for his careful supervision of the work. I am also indebted to Dr. Dorothea Egleston Smith for the preparation of some phosphorus-free molybdic acid used in the micro-chemical tests. The material was collected by Dr. Tennent at Tortugas in the summer of 1925. Mature, uninseminated eggs of *Lytechinus variegatus* were fixed in Champy-Kull. Some of the eggs from the same females were inseminated with sperm from males of the same species and fixed (1) in Champy-Kull twenty minutes after insemination, and (2) in strong Flemming (Lee, 1924, p. 35) twenty-one minutes after insemination. The eggs were brought from Tortugas in 80 per cent alcohol, and turned over to me for study in December 1926. After dehydration and clearing in xylol, the eggs were embedded in 52° to 55° C. paraffin and sectioned at 3μ. The Champy-Kull material was mounted with a row of fertilized and a row of unfertilized eggs on each slide, so that the staining and micro-chemical treatment were identical for the material to be compared. No uninseminated eggs had been fixed in Flemming, so that a comparison of stainable inclusions was made between the eggs which had escaped fertilization and those which had been successfully inseminated. A study of the unfertilized eggs of this material and uninseminated *Lytechinus* eggs of a different set, but similarly fixed, showed that failure to fertilize was due to chance and not to any peculiarities of their stainable cell components.

The following staining methods were used after Champy-Kull fixation:

1. Heidenhain's iron hematoxylin method (longer process) (Lee, 1924, p. 149).
2. Benda's alizarin method as modified by Meves and Duesberg (1908).
3. Champy-Kull acid fuchsin, toluidin blue and aurantia method (Lee, 1924, p. 32) with the following modifications: (*a*) slides were mordanted for 2 minutes in 2 per cent potassium bichromate, blotted with filter paper and treated immediately with acid fuchsin. (*b*) Treatment with toluidin blue was shortened to 45 seconds. (*c*) Differentiation with aurantia was prolonged to from 1 to 2 minutes.
4. Graser's basic fuchsin method (Lee, 1924, p. 169).

After Flemming:

1. Heidenhain's iron hematoxylin method.
2. Graser's basic fuchsin method.
3. Flemming's orange G method (Lee, 1924, p. 177). An aqueous solution of gentian violet was used and the treatment with orange G was shortened to about 30 seconds.

Grübler's dyes were used in all cases.

The test for organic phosphorus of Bell and Doisy (1920) as employed by Gardiner (1927) was used.

OBSERVATIONS

STAINABLE SUBSTANCES PRESENT IN THE EGG BOTH BEFORE AND AFTER FERTILIZATION

Macrosomes

The most numerous of the stainable granules present in *Lytechinus* eggs are the "macrosomes" or alveolar spheres of Wilson. These spheres always stain slightly darker than the hyaloplasm in which they lie. After Champy-Kull fixation, they stain gray with hematoxylin (fig. 7). With acid fuchsin, toluidin blue and aurantia or with basic fuchsin and methylen-blue they are pinkish blue. With Benda they stain pale violet or yellowish pink, depending on the length of differentiation. After Flemming fixation, the macrosomes stain pinkish yellow with orange G. With hematoxylin, they become gray, and with basic fuchsin pinkish blue, as after the Champy-Kull stain. In the unstained egg they are indistinguishable from other cell inclusions.

In the unfertilized egg these macrosomes appear in irregular groups scattered throughout the cytosome (fig. 3). In the prophases, represented in the fertilized eggs which I have studied, there is an accumulation of the hyaline substance around the nucleus, especially in the astral regions. The macrosomes lie between the paths of this hyaloplasm which radiate from the astral centers (fig. 4).

Chondriosomes

Typical chondriosomes are present as numerous, small, highly refractive granules scattered uniformly throughout the cytoplasm. They have sharp, clear outlines and in most cases are spherical, although occasionally a granule appears to have a lump on its surface. After Champy-Kull fixation, the chondriosomes stain red with acid fuchsin (figs. 1 and 2), deep violet with Benda (figs. 3 and 4), and black with hematoxylin (figs. 7 and 8). They give a positive reaction to the test for organic phosphorus. In these eggs they are also preserved by Flemming as may be seen from the hematoxylin preparations of figures 5 and 6.

Large Oxyphilic Phospholipid Spheres

Neither the Benda nor the acid fuchsin stain is specific for chondriosomes, for both lipins and phospholipins are stainable by these methods. Miller and Smith (1930) were able by extraction of the crystal violet to show that in *Echinometra* the stain was persistent in granules of phospholipin which were contained in a surrounding medium of lipin.

In *Lytechinus* there are numerous large, refractive spheres scattered throughout the egg which stain similarly to the chondriosomes in acid fuchsin (figs. 1 and 2); in Benda (figs. 3 and 4), and in hematoxylin (figs. 7 and 8). These spheres do not blacken with osmic acid. They show a distinct blue in the phosphorus test. There are granules which range in size from the largest of these spheres to the small chondriosomes. With the methods employed, no differences were noted between the staining reactions of these large spheres and those of the small chondriosomes and no definite proof that they differ in composition has been obtained. However, from the size of the large spheres and from the fact that the stains are known to be unspecific, it seems possible that the larger spheres may be yolk.

FAT

Spherical globules, which have been blackened by the osmic acid of the fixing fluid after the manner typical of fats, are of rare occurrence in both the Champy-Kull and the Flemming material. Some eggs contain no such globules, while others may contain three or four. These globules are black in both unstained and stained eggs. One of them is shown in the Champy-Kull acid fuchsin preparation of figure 1. From their behavior it seems evident that these globules are of a fatty nature.

BASOPHILIC GRANULES

Figure 9, which is drawn from an unfertilized egg stained with basic fuchsin after Flemming, shows a set of highly refractive granules which stain a brilliant red. These granules are spherical and in section have clear, sharp outlines. They vary considerably in size. A few appear in the central region of the cell, but a larger number occur near the periphery.

Figure 10 is drawn from a fertilized egg after the same fixation and staining. The same set of granules appears with a similar arrangement after staining with basic fuchsin. In addition, however, a number of granules appear in the nucleus, which is in prophase. These nuclear spheres stain with basic fuchsin like the extra-nuclear granules. Their appearance in the nucleus is in contrast to the condition in the resting nucleus of the unfertilized egg which shows the presence of no basophilic substance. With hematoxylin the nuclear spheres are black. In order to confirm the belief that the extra-nuclear granules are also stained by hematoxylin, a slide was stained first in hematoxylin and then in basic fuchsin. After this treatment no granules showed any red-staining reaction.

That these granules are also preserved by Champy-Kull may be determined by staining with basic fuchsin after this fixation. Although the staining reaction is not so brilliant as after Flemming, the granules which do take a pale red stain have an arrangement unmistakably the same as that of the basophilic granules of the other fixation. With acid fuchsin the extra-nuclear granules are indistinguishable from the macrosomes. In the nucleus of the fertilized egg, they take the same stain as the ground substance. The same is true of the Benda preparations (figs. 3 and 4). With hematoxylin they stain black.

That the intra-nuclear granules are of a phospholipid nature is indicated by their positive reaction to the test for orthophosphate. It is believed that the extra-nuclear granules also give a positive reaction, although the difficulty of distinguishing them from other phosphorus-containing inclusions renders this conclusion uncertain.

The similarity in staining reaction of the extra-nuclear basophilic granules and those of the basichromatin of the prophase nucleus suggests the possibility of their nuclear origin. The study of this material, however, gives no evidence of any morphological or physiological change in the extra-nuclear granules concomitant with the appearance of the basichromatin in the nucleus. In order to draw any definite conclusions as to the relation of the basophilic bodies to nuclear phenomena, a larger number of stages than are available in this material would have to be examined.

STAINABLE SUBSTANCES WHICH DISAPPEAR UPON FERTILIZATION

Figure 1 shows a portion of an unfertilized egg fixed in Champy-Kull and stained with acid fuchsin, toluidin blue and aurantia. An aggregation of small red granules in the cortical region is quite noticeable. Some of these are clearly spherical with sharp, smooth outlines; others are somewhat flattened or show bulges in their outlines. Figure 2 shows a fertilized egg similarly fixed and stained. A much smaller number of fuchsinophile granules appears in the cortex. This same change in the number of small stainable granules is evident from a comparison of figures 7 and 8, which were drawn from eggs stained by hematoxylin after Champy-Kull.

This set of cortical granules is always stained by hematoxylin like the other granules throughout the cytosome. With acid fuchsin, however, their reaction is slightly different. With a short treatment with aurantia they appear to be more faintly stained than the other granules. That this is not due to size differences is indicated by the fact that other equally small granules in the egg are well stained when these are hardly distinguishable. A few peripheral granules, including some small and some large ones, are always well stained with this shorter differentiation. They seem to correspond to the granules which remain after fertilization, as is shown in figures 2 and 8. With a longer differentiation with aurantia (2 min.) no difference in the staining of the peripheral and other granules is noticeable (fig. 1).

This set of cortical granules fails to show any reaction to the phosphorus test. The few peripheral granules which do show the blue color after this test belong quite clearly, from their size, shape and arrangement, to the class of phospholipid bodies which remain in the egg after fertilization.

With Benda's stain after Champy-Kull fixation (fig. 3), this peripheral set of granules does not take the deep violet stain as do the substances which are in the egg both before and after fertilization. This observation seems to indicate that the specificity of crystal violet for phospholipins is greater than that of acid fuchsin.

Figures 5 and 6 were drawn from unfertilized and fertilized eggs respectively which had been stained with hematoxylin after Flemming fixation. The striking aggregation of stainable substance in the cortex of the unfertilized egg and its absence in the fertilized egg are evident. The irregular form of the individual masses which comprise this layer indicates the possibility that it is a colloidal substance precipitated by the fixing fluid. That it is a lipin rather than a phospholipin is suggested by its negative reaction to the test for phosphorus.

A comparison of this cortical substance with the substance stained by acid fuchsin and by hematoxylin after Champy-Kull fixation (figs. 1 and 7) shows several differences between the two. In the first place, it is evident that a much larger amount of cortical material has been preserved by Flemming's fluid. A greater irregularity in the form of individual granules also is noticeable in the Flemming material.

The difference in methods of fixation may be the cause of some differences in the appearance of a precipitation product. However, in view of the greater amount of cortical substance stainable after fixation with Flemming and of the fact that the contrast in cortical appearance before and after fertilization is greater in the Flemming than in the Champy-Kull material, it seems probable that two different substances, each concerned in some way with the fertilization process, have been preserved by the two different fixations.

SUMMARY

1. In the egg of *Lytechinus variegatus*, after Champy-Kull fixation, certain cytoplasmic substances may be demonstrated both before and after fertilization. These are: (*a*) macrosomes and hyaloplasm, (*b*) chondriosomes, (*c*) large oxyphilic, phospholipin spheres, (*d*) fat droplets, (*e*) extra-nuclear basophilic granules.

2. After the same fixation, a cortical substance in the form of lipid granules is present in the mature, unfertilized egg, but disappears upon fertilization.

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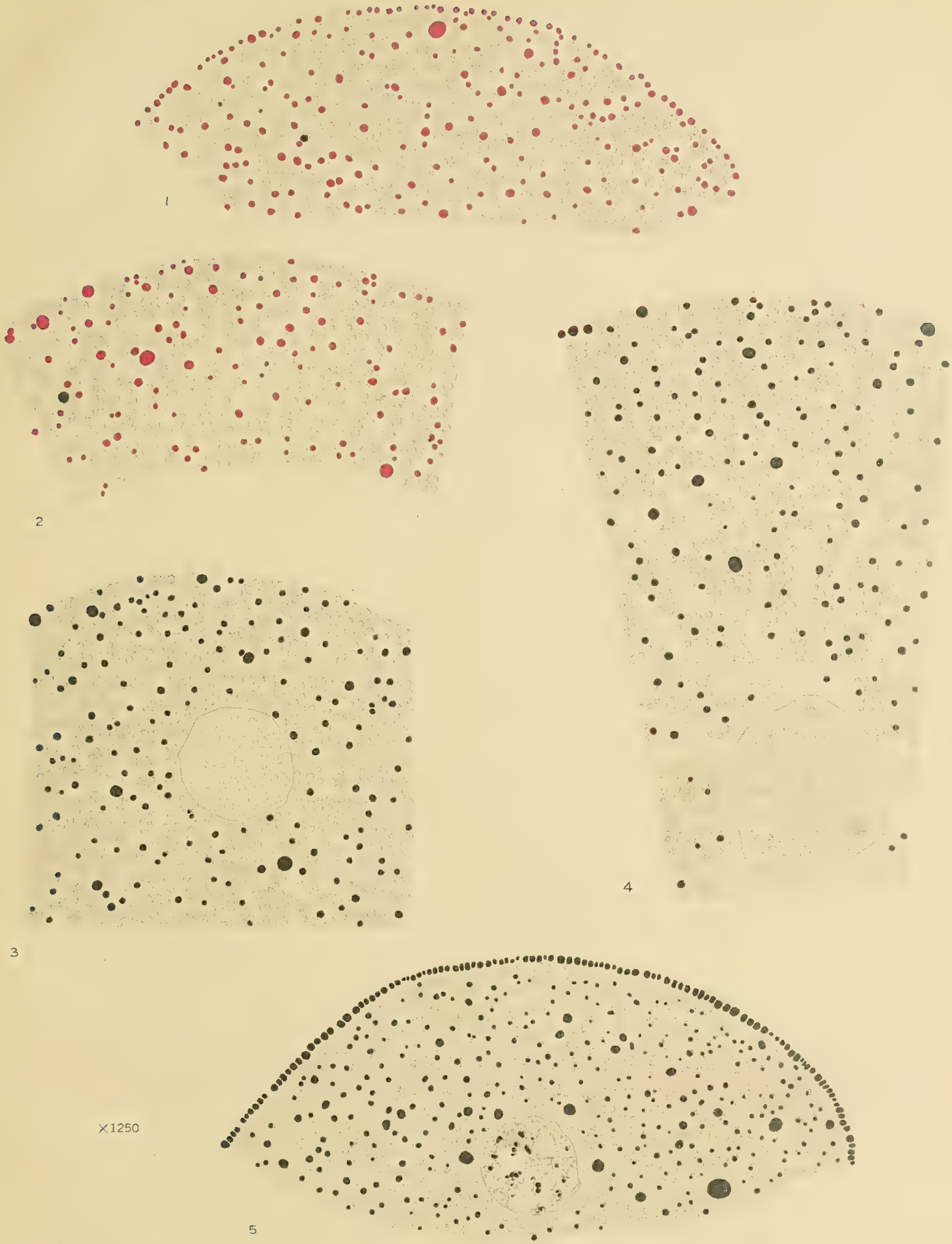
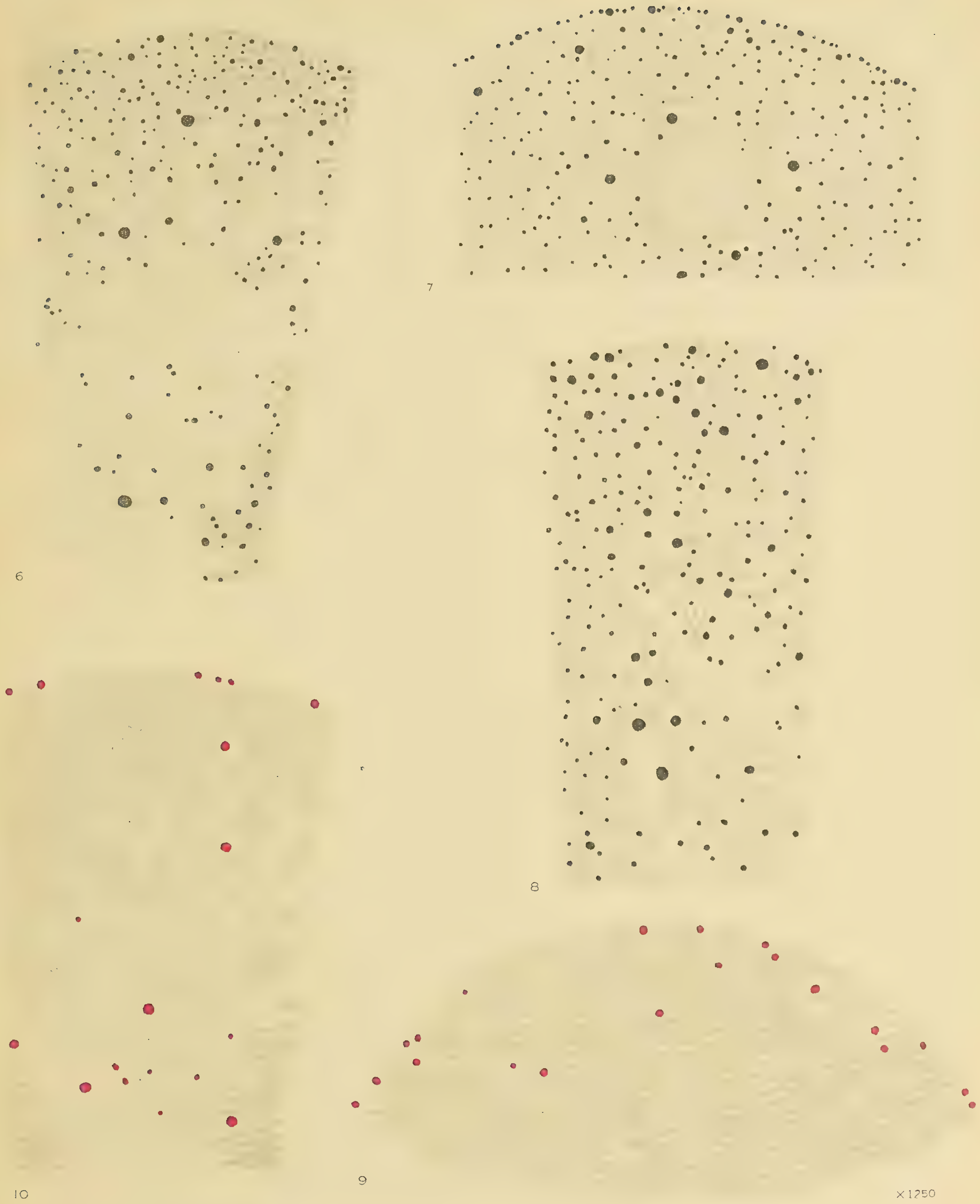
EXPLANATION OF FIGURES

All figures were drawn 19.5 centimeters below table level by means of an Abbe camera lucida, using a Zeiss 2.0 mm. oil immersion objective and a Zeiss compensating ocular No. 8, resulting in a total magnification of 2500 diameters. They were reduced one-third in reproduction, making a final magnification of 1667 diameters.



FORMED COMPONENTS AND FERTILIZATION IN THE EGG OF THE SEA URCHIN, LYTECHINUS VARIEGATUS

Fig. 1. Unfertilized egg. Champy-Kull, acid fuchsin, toluidin blue and aurantia.
Fig. 2. Twenty minutes after insemination. Champy-Kull, acid fuchsin, toluidin blue and aurantia.
Fig. 3. Unfertilized egg. Champy-Kull, Benda.
Fig. 4. Twenty minutes after insemination. Champy-Kull, Benda.
Fig. 5. Unfertilized egg. Flemming, hematoxylin.



FORMED COMPONENTS AND FERTILIZATION IN THE EGG OF THE SEA URCHIN, LYTECHINUS VARIEGATUS

Fig. 6. Twenty-one minutes after insemination. Flemming, hematoxylin.
Fig. 7. Unfertilized egg. Champy-Kull, hematoxylin.
Fig. 8. Twenty minutes after insemination. Champy-Kull, hematoxylin.
Fig. 9. Unfertilized egg. Flemming, basic fuchsin and methylen blue.
Fig. 10. Twenty-one minutes after insemination. Flemming, basic fuchsin and methylen blue.

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